MECHANISMS OF GLUCOCORTICOID TYPE II RECEPTOR REGULATION AND ACTIVATION IN BRAIN

By

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Glucocorticoids have been shown to have profound effects on the mammalian nervous system, most of which have been shown to result from a receptor-mediated change in concentration or activity of key proteins. This change is a consequence of an action of the glucocorticoid-receptor complex on the molecular machinery that controls gene expression. Upon binding of a steroid by the receptor, it undergoes a temperature-dependent activation, correlated with changes in a number of receptor properties, before it can effectively interact with its nuclear acceptor sites.

The surface hydrophobicity of the glucocorticoid receptor in brain cytosol was examined using hydrophobic interaction chromatography. There was a clear increase in hydrophobicity upon activation, a finding with important implications for the interactions between activated receptors and numerous nuclear components.

The successful purification of the glucocorticoid receptor allowed for an investigation of activation relatively free of cytosolic factors that could interfere with, or contribute to, the process. Although activation of unpurified receptors led to a 50-fold increase in DNA-cellulose binding, there was no increase when purified receptors from brain or liver were subjected to identical activation conditions. However, other changes in purified receptors associated with activation, including an increase in surface hydrophobicity, implied the possibility of a multistep process.

Unoccupied glucocorticoid receptors can exist in a non-steroid-binding or down-regulated state which can be up-regulated to a steroid-binding state upon addition of sulfhydryl reducing reagents. An investigation of the hydrodynamic and biochemical nature of the up- and down-regulated receptors revealed that they exhibited the same sedimentation properties, but differed in surface hydrophobicity. In addition, the up-, but not the down-, regulated form proved susceptible to irreversible inactivation by some sulfhydryl reactive reagents, allowing for a determination of the respective populations of the two forms in cytosol or homogenate.

Sedimentation of the unoccupied receptor was examined by post-labeling procedures and was found to differ in the presence (9-10 S only) or absence (9-10 & 4-5 S) of molybdate. This effect of molybdate was mimicked somewhat by heat-stable cytosolic factors.

CHAPTER I

GENERAL INTRODUCTION

Glucocorticoid steroid hormones are known to have profound metabolic, neuroendocrine and behavioral effects in the mammalian brain. Glucocorticoids, in addition to regulating ACTH secretion (for review see Keller-Wood and Dallman, 1984), produce alterations in mood, changes in detection and recognition of sensory stimuli, changes in sleep and in the extinction of previously acquired habits, and alterations of electric activity of the brain (for reviews see Raynaud et al., 1980; McEwen et al., 1982; Schraa and Dirks, 1982; Luttge, 1983; De Kloet, 1984; Reese and Gray, 1984; Meyer, 1985). Since most of these reviews were written, many new reports concerning the role of glucocorticoids in the nervous system have appeared. A number of studies have further investigated the localization of glucocorticoid binding in different regions of the central nervous system including the spinal cord (Clark et al., 1981; Duncan and Stumpf, 1984; Orti et al., 1985), the locus ceruleus (Krasuakaya, 1982), the hypothalamus (Kato, 1983; Ribarac-Stepic et al., 1984), the caudate-putamen (Defiore and Turner, 1983), the hippocampus (De Kloet et al., 1984), or a variety of different brain regions (Tornello et al., 1981; Dordevic-Markovic et al., 1983; Alexis et al., 1983; Sarrieau et al., 1983, 1984; Birmingham et al., 1984; Ribarac-Stepic et al., 1984).

Many recent studies have focused on the role of glucocorticoids in the development of the nervous system. Receptor binding studies in the

developing brain imply an early sensitivity to glucocorticoids (Kitraki et al., 1984), particularly in the cerebellum (Pavlik and Buresova, 1984). The general in vivo effect of glucocorticoids on brain growth and development appears to be one of suppression (Slotkin et al., 1982; Doerner, 1983; Meyer, 1983; DeKosky et al., 1984; Pavlik et al., 1984) although adrenalectomy of young rats was shown to result in hypomyelination of the central nervous system (Preston and McMorris, 1984) indicating that the effects of glucocorticoids on neural development are complex. Using a brain cell culture system, Stephens (1983) reported that glucocorticoids (cortisol) stimulate rather than inhibit the processes of myelinogenesis and brain development. Investigations of the development of synapses between cholinergic neurons and retina muscles in a cell culture system led to the hypothesis by Puro (1983) that glucocorticoids regulate the development of mechanisms which couple neuronal depolarization with release of neurotransmitter. Lending some potential support to this hypothesis are the findings of Leeuwin et al. (1983) who reported that glucocorticoids induced significant changes in the morphology of synaptic vesicles of cholinergic (phrenic) nerve terminals. Smith and Fauquet (1984) reported that glucocorticoids indirectly stimulate adrenergic differentiation in cultures of migrating and premigratory neural crest by selectively enhancing catecholaminergic properties in neural crest cells that had already been exposed to an appropriate signal of another kind. Finally, a variety of behavioral, biochemical and morphological teratologies of the central nervous system are known to be induced by glucocorticoids (Bohn, 1984; Pratt et al., 1984).

Glucocorticoids have profound and complex effects on brain metabolism. Podvigina et al. (1983) reported that a variety of glucocorticoids inhibited respiration and phosphorylation and increased glycolysis in slices and homogenates of rat cerebral cortex, hippocampus and hypothalamus. In agreement with glucocorticoid binding data, these workers found hippocampus to be most sensitive and cortex to be least sensitive. Interestingly, Kamenov (1981) has suggested that the degree to which glucocorticoids affect respiration may itself be under the indirect control of the nervous system and may vary seasonally in some animals. These and many other metabolic functions in brain are modulated by glucocorticoids via an impressive number of enzymes. Patel et al. (1983) showed recently that corticosterone treatment resulted in an increase in glutamine synthetase activity in rat brain. These findings were confirmed in mouse brain cell culture by Stephens (1983) who found that cortisol also increased the activity of 2',3'-cyclic nucleotide phosphohydrolase while it caused a decrease in arylsulfatase A activity (thought to be important in myelinogenesis). Earlier work regarding the role of glucocorticoids in glia is reviewed in McEwen (1984). The activity of glycerol 3-phosphate dehydrogenase, another enzyme thought to be involved in myelination and present in high concentrations in oligodendrocytes, was recently shown to be reduced in rat brain after adrenalectomy. Rat brain citrate synthetase activity, on the other hand, has been recently shown to undergo an actinomycin D-reversible decrease upon administration of cortisol (Sharma and Patnaik, 1984). These same workers reported earlier that cortisol differentially regulates the activity of malate dehydrogenase isoenzymes in rat brain (Sharma and Patnaik, 1982). Complex results have been

obtained for tyrosine aminotransferase activity in brain as well. Mishunina and Babicheva (1981) reported that a single injection of cortisol to rats caused a reduction of tyrosine aminotransferase activity in the hypothalamus and medulla oblongata while causing an increase in the activity in the hippocampus. Glucocorticoid injection has also been shown to increase arginase activity in guinea pig brain (Bjelakovic and Nikolic, 1983) and to "modulate" ornithine decarboxylase activity in rat brain (De Kloet et al., 1983). Interestingly, tyrosine hydroxylase activity in the superior cervical ganglion was shown to be increased by injections of the synthetic glucocorticoid, dexamethasone, but not by endogenous glucocorticoids (corticosterone or cortisol) regardless of dose (Sze and Hedrick, 1983). Eranko et al. (1982), using fluorescent histochemical techniques, reported that injection of newborn rats with cortisol caused a great increase in the number of the small, intensely fluorescent cells and the appearance of similar small cells with intense immunohistochemical reactions for tyrosine hydroxylase (TH), dopamine-B-hydroxylase (DBH) and phenylethanolamine (norepinephrine) N-methyltransferase (PNMT) in the superior cervical ganglion. At the same time, the epinephrine content and the PNMT activity of the ganglion greatly increased, while no significant changes were observed in the dopamine or norepinephrine content or TH or DBH activity. PNMT expression has also been shown to be influenced by glucocorticoids in cells derived from the neural crest (Bohn, 1983).

Single acute doses of dexamethasone have been recently shown to affect the levels of monoamines and their metabolites in rat brain (Rothschild et al., 1985). These researchers reported significant increases in dopamine levels in the hypothalamus and nucleus accumbens

of dexamethasone-treated rats when compared with saline-treated rats, whereas no significant effect of dexamethasone on dopamine levels in frontal or striatal brain areas was found. These workers also found that dexamethasone led to a significant increase in serotonin in hypothalamus, whereas dexamethasone caused a significant decrease in serotonin in the frontal cortex. These findings may help to explain the observations that corticosteroids lower prolactin levels and can induce psychiatric disturbances as well as the finding that affectively ill patients with high post dexamethasone cortisol levels are frequently psychotic.

Other recent findings of relevance to the effects of glucocorticoids on neurotransmitter systems include the report by Mishunina and Kononenko (1983) that a single injection of cortisol increased the uptake of gamma-aminobutyric acid (GABA) by synaptosomes of hippocampus, hypothalamus and cerebral cortex. However, in vitro studies by these same workers resulted in a glucocorticoid-dependent increase in GABA uptake by only the hypothalamus synaptosomes, indicating differential mechanisms of glucocorticoid action in these different brain regions. Haidarliu et al. (1983) reported that perfusion of the rat neostriatum with dexamethasone after stereotaxic injections of [3H]-labeled dopamine into the caudate nucleus increased basal but inhibited potassium-stimulated [3H]dopamine release and yield of metabolites. Slotkin et al. (1982) reported that neonatal exposure to dexamethasone resulted in alterations in norepinephrine synthesis and turnover in both central and peripheral sympathetic neurons. Further demonstration of the relationship between glucocorticoids and central neurotransmitter systems is evident from studies reporting a decrease in the

corticosterone binding capacity of hippocampus after administration of 6-hydroxydopamine (Weidenfeld et al., 1983) or 5,7-dihydroxytryptamine (Siegel, 1983). Similarly, the possible role of medial forebrain bundle catecholaminergic fibers in the modulation of glucocorticoid negative feedback effects has been further elucidated by investigating the effects of 6-hydroxydopamine treatments in rat brains (Feldman et al., 1983). In addition to effects on transmitter uptake and synthetic and degradative enzyme activity, some of the effects of glucocorticoids on catecholaminergic function are known to be via catecholaminergic receptors (see review by Davies and Lefkowitz, 1984).

Many of the actions of glucocorticoids outside the brain, such as their effects on lipolysis, gluconeogenesis, glycogenesis and glycogenolysis, are regulated primarily by cAMP or hormones acting through cAMP leading to the notion that glucocorticoids have potentiating (permissive or synergistic) effects on the action of many hormones that act through cAMP as the intracellular second messenger (for review see Liu, 1984). Such wide-ranging effects of glucocorticoids on the effects of these other hormone systems, thought to be via the regulation of the autophosphorylation of cAMP-dependent protein kinase, will likely be shown to play an important role in the glucocorticoid modulation of neural function as well.

A recent finding of potential importance to the field of epilepsy research involves the discovery that natural, but not synthetic, glucocorticoids modulate the binding of [3H]muscimol, a GABA agonist, in crude synaptosomal membranes and in brain sections of rats (Majewska et al., 1985). In general, nanomolar concentrations concentrations of corticosterone and pregnenolone-sulfate enhanced mucimol binding in

cerebral cortex, cerebellum, thalamus and hippocampus via an apparent increase in the affinities of GABA receptors. More recently, two metabolites of the steroid hormones progesterone and deoxycorticosterone, were shown to be potent barbiturate-like ligands of the GABA receptor-chloride ion channel complex (Majewska et al., 1986). At low concentrations both steroids inhibited binding of the convulsant t-butylbicyclophosphorothionate to the GABA-receptor complex and increased binding of benzodiazepine flunitrazepam. In addition, chloride uptake into isolated brain vesicles was stimulated and the inhibitory actions of GABA in cultured rat hippocampal and spinal cord neurons were potentiated. These findings may help to explain the ability of certain steroid hormones to rapidly alter neuronal excitability (see below) and may provide a mechanism for the anesthetic and hypnotic actions of naturally occurring and synthetic anesthetic steroids.

The effects of glucocorticoids on the electrical activity of neurons has been studied at the level of single neurons by the method of microiontophoresis. This technique has been used to study glucocorticoid-induced changes in activity of neurons in a variety of brain regions including hypothalamus (Mandelbrod et al., 1981), medial septal area (Papir-Kricheli and Feldman, 1981), reticular formation (Avanzino et al., 1983) and hippocampus (Belyi et al., 1982). In most of these studies glucocorticoids were found to inhibit some neurons, excite some neurons and have no effect on still other neurons in the same brain region, although the ratios of the three groups varied between regions. Peripherally, the effects of glucocorticoids on neuromuscular function have also been investigated. These effects are generally facilitatory

(see review by Hall, 1983), although there have been reports to the contrary (Korneeva and Emelyanov, 1981).

The role of glucocorticoids in the regulation of physiological reactions to stress has been widely studied (for review see Munck et al., 1984; Sapolsky et al., 1986) and at least some of these stress-related functions of glucocorticoids are known to involve the central nervous system. One example that has received a good deal of attention recently involves the effects of glucocorticoids on stress-induced analgesia. Adrenalectomy, hypophysectomy and/or glucocorticoid treatment have all been shown to have significant effects on the stress-induced (cold water, footshock, etc.) inhibition of pain responsiveness. Markel et al. (1984) reported that adrenalectomy significantly reduced the threshold for footshock sensitivity in rats and that steroid replacement restored, or even potentiated, this pain sensitivity. Similarly, Mousa et al. (1983) reported that analgesia induced in rats by cold-water swim stress and measured by the tail-flick and hot-plate methods was significantly antagonized after IP pretreatment for three days with dexamethasone. These investigators go on to suggest that since naloxone also attenuated the analgesia developed by the cold-water swim stressor, corticosteroids may have a role in modulating stress-induced analgesia via the endogenous opiate system. Evidence of this was presented recently by Terman et al. (1984) who used inescapable foot shock as a stressor and the tail-flick test as a measure of analgesia. These workers, using multiple doses of glucocorticoids, found that lower doses potentiated and higher doses significantly reduced stress analgesia in both sham and hypophysectomized animals. Despite some evidence to the contrary (Baron and

Gintzler, 1984), these results, along with the finding that nonopiod analgesia is unaffected by dexamethasone (Lewis et al., 1980) and that corticosterone exerts the same biphasic dose-dependent effects on morphine analgesia (Terman et al., 1985), suggest an opiod action for glucocorticoids, perhaps at the receptor level. Of some potential relevance to this topic is a report that adrenalectomy significantly potentiated and corticosterone administration attenuated stress-induced decreases in norepinephrine contents in the hypothalamus and thalamus, and the increases in 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (a principle metabolite of norepinephrine) levels in the hypothalamus, amygdala and thalamus (Nakagawa et al., 1983).

The idea that glucocorticoids, via the adrenocortical axis, may have an important role in the process of aging has been examined in depth recently (Sapolsky et al., 1986). It has been shown that the aged male rat is impaired in terminating the secretion of glucocorticoids at the end of stress. This hormonal excess is thought to be due to degenerative changes in a region of the brain which normally inhibits glucocorticoid release; the degeneration, in turn, appears to be caused by cumulative exposure to glucocorticoids (Sapolsky and McEwen, 1985; Sapolsky, 1986). Together, these effects form a feed-forward cascade in the aging subject with potentially serious pathophysiological consequences.

In addition to the above findings regarding the role of glucocorticoids in the nervous system, glucocorticoids are clinically important in the field of neurosurgery, most notably for the treatment of various kinds of brain edema related to intracranial tumors, cerebral infarctions and head injuries (Bouzarth and Shenkin, 1974;

Sugiura et al., 1980; Yu et al., 1981).

Although there is limited evidence of glucocorticoid binding directly to synaptic (Towle and Sze, 1983) and other (Duval et al., 1983) membranes, most of the glucocorticoid effects amenable to experimentation have been shown to result from a receptor-mediated change in concentration or activity of key proteins. This change is a consequence of an action of the glucocorticoid-receptor complex on the molecular machinery that controls gene expression. Recent advances such as recombinant-DNA and monoclonal-antibody techniques have offered new ways of exploring this machinery. Recently, a monoclonal antibody against the rat liver glucocorticoid receptor in combination with the indirect immunoperoxidase technique has made it possible to demonstrate glucocorticoid receptor-immunoreactive nerve and glial cell nuclei all over the tel- and diencephalon of the male rat (Fuxe et al., 1985). Since the glucocorticoid receptor qualifies as one of the few proteins known to control gene expression in eukaryotes, it is not surprizing that glucocorticoids have become a fashionable tool for the molecular biologist (for reviews see Ringold et al., 1983; Lan et al., 1984; Hager et al., 1984; Rousseau, 1984a; Scheidereit et al., 1986). A major step in the understanding of the structure and function of the glucocorticoid receptor was made recently when the primary structure of a functional human glucocorticoid receptor cDNA was identified and subsequently cloned (Govindan et al., 1985) and expressed in vitro (Hollenberg et al., 1985). The focus of the following review and the proposed research, however, will be the receptor molecule itself and the changes in this molecule brought about by the binding of a steroid ligand which enable it to interact with the machinery that controls gene expression.

Although little has been said in this brief review regarding the structure or function of the receptors in brain for other classes of steroids (i.e. estrogens, progestins, androgens), it is particularly relevant to briefly note recent findings regarding the so called mineralocorticoid, or "type I", receptor in neural tissues. The relevance of mineralocorticoid receptors to glucocorticoid actions in neural tissues stems first from the fact that [3H]aldosterone has been shown to bind to glucocorticoid, or "type II", receptors as well as type I receptors in brain cytosol (Anderson and Fanestil, 1976) by virtue of biphasic Scatchard plots for [3H]aldosterone, but not [3H]dexamethasone. Later reports indicated that inclusion of the new synthetic glucocorticoid RU 26988 (unlabeled), which binds to type II but not type I receptors or corticosteroid binding globulin (Mogiulewsky and Raynaud, 1980), linearizes the [3H]aldosterone Scatchard plot (Beaumont and Fanestil, 1983; Emadian et al., 1986), leaving only a high-affinity component assumed to be the type I receptor. A second point of relevance involves the fact that in addition to aldosterone, corticosterone has a high affinity for the type I receptor (Krozowski and Funder, 1983), which has led some to believe that corticosterone may be a major, if not the primary, ligand for type I receptors in brain. This topic is of great concern to those investigating glucocorticoid actions in the nervous system and is currently the subject of investigation by others in this lab. It should be noted, however, that synthetic glucocorticoids, such as dexamethasone or triamcinolone acetonide, have a much lower affinity for the type I receptor and usually possess "purer" glucocorticoid properties than corticosterone as determined by their physiological actions in peripheral tissues

(Meyer, 1985). In addition to their high specificity for type II glucocorticoid receptors, other advantages of using these synthetic glucocorticoids include the fact that their rate of dissociation from the receptor is considerably slower than that of natural glucocorticoids and have an insignificant affinity for corticosteroid binding globulin. For these and other reasons, the research reported here was carried out using dexamethasone and/or triamcinolone acetonide exclusively, and therefore all further references to "glucocorticoid receptors" will imply type II receptors for adrenal steroids unless otherwise indicated.

Major areas of glucocorticoid receptor research that are of particular relevance to the glucocorticoid modulation of nervous system function include the following: 1) The stabilization and potential mechanisms of up- and down-regulation of unoccupied glucocorticoid receptors. 2) Activation of the glucocorticoid-receptor complex to the nuclear-binding form and subsequent interaction with the genome. 3) Purification of the glucocorticoid receptor and subsequent detailed physicochemical analysis.

CHAPTER II

HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF VARIOUS FORMS OF THE OCCUPIED AND UNOCCUPIED GLUCOCORTICOID RECEPTOR

Introduction

Hydrophobic interactions basically result from the adherence of non-polar solutes or groups in aqueous solution, resulting, consequently, from their repulsion from the strong polar-polar interactions of water molecules. Like its ionic properties, a molecule's surface hydrophobicity depends upon its primary structure. A change in the surface hydrophobicity of glucocorticoid-receptor complexes associated with activation has been suggested by workers reporting an increase in the partitioning of these complexes after activation into the polyethylene glycol (PEG) layer in an aqueous dextran-PEG two phase system (Andreasen, 1978 and 1982; Andreasen and Mainwaring, 1980) and by Luttge et al. (1984d) who, in addition to an increase in partitioning into PEG, found a large increase in the fractional adsorption of [3H]triamcinolone acetonide-receptor complexes to glass fiber (Whatman GF/C) filters following activation. This putative increase in hydrophobicity does not appear to be unique to the glucocorticoid receptor, since similar transformations following activation have been reported recently for estrogen (Gschwendt and Kittstein, 1980; Murayama and Fukai, 1983), androgen (Bruchovsky et al., 1981) and progesterone (Lamb and Bullock, 1983) receptors. Although the functional significance of this increase in hydrophobicity is unknown,

it may contribute to the increased binding affinity of the activated receptor for DNA. Increasing the hydrophobicity of the receptor may also increase its rate of permeation through the nuclear membrane.

Hydrophobic interactions have also been reported to account for the high affinity binding of glucocorticoids to their receptors (Wolf et al., 1978; Alfsen, 1983; Eliard and Rousseau, 1984). According to these workers, binding requires both sides of the steroid to be enveloped by the receptor.

The influence of hydrophobic interactions on the structure and steroid-binding properties of glucocorticoid receptors has recently been investigated by means of chromatography on Sephacryl S-300 or Lipidex 1000 or by incubation with charcoal or phospholipase C (Bell et al., 1986). These workers suggest that receptor activation is preceded by structural changes associated with the loss of a lipid factor from the complex. They also reported that non-polar steroid antagonists, and lipophilic compounds such as phenothiazines, bound to secondary, hydrophilic sites on the receptor and exerted allosteric effects on the primary steroid-binding site implying that hydrophobic interactions may be important determinants of the structure and properties of glucocorticoid receptors.

One particularly useful method for investigating the hydrophobic characteristics of steroid receptors is hydrophobic interaction chromatography. Typically this involves characterizing the chromatographic behavior of the receptor in question on a series of hydrophobic matrices of decreasing polarity. Bruchovsky et al. (1981) investigated the hydrophobic properties of the rat prostatic nuclear androgen receptor using gamma-amino-alkyl derivatives of agarose with varying

alkyl substituent lengths. These workers found the adsorption of receptor to the modified agarose gels increased with the length of the alkyl substituent. However, the presence of the terminal amino group in these gels introduces the possibility of ionic effects which could complicate the interpretation of the hydrophobic interactions (Shaltiel, 1974). More recently, Lamb and Bullock (1983) investigated the hydrophobic properties of the rabbit uterine progesterone receptor using a series of alkyl agarose columns of increasing alkyl chain length (no terminal amino group). No such studies have been conducted on glucocortcoid receptors.

In addition to gaining further knowledge regarding the process of receptor activation, more detailed hydrophobic interaction studies of both activated and unactivated forms of the glucocorticoid-receptor complex, as well as the non-steroid bound form of the receptor, are likely to provide valuable information concerning the physicochemical properties of each of these receptor forms. Such studies might also provide potentially useful means by which partial purification of these different receptor forms can be achieved.

Materials and Methods

Chemicals, Steroids and Isotopes

[6,7-3H]Triamcinolone acetonide, or 9a-fluoro-11b,16a,17a,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide, ([3H]TA, specific activity = 37 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Sephadex G-25 (fine) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid were courtesy of Research Organics (Cleveland, OH). Sodium molybdate (Na2MoO4), calf thymus DNA-cellulose, glycerol,

PPO (2,5-diphenyloxazole) and dimethyl POPOP (1,4-bis[2(4-methyl-5-phenyloxazoyl)]benzene), phenyl agarose and alkyl agaroses were all purchased from Sigma Chemical Co. (St. Louis, MO). Scinti Verse II was purchased from Fisher, Inc. (Fair Lawn, NJ). All other chemicals and solvents were reagent grade.

Animals

All studies used female CD-1 mice (Charles River Laboratories, Wilmington, MA) that were subjected to combined ovariectomy and adrenalectomy approximately 1 week prior to each experiment in order to remove known sources of endogenous steroids. Both operations were performed bilaterally via a lateral, subcostal approach under barbiturate anesthesia, and mice were given 0.9% NaCl (w:v) in place of drinking water. On the day of the experiment mice were anesthetized with ether and perfused slowly through the heart with ice-cold HEPES-buffered saline (20-30 ml, isotonic, pH 7.6).

Cytosol Preparation and Steroid Binding

Brains were removed from the perfused animals and homogenized (2 x 10 strokes at 1000 rpm) in 4 volumes of ice cold Buffer A (20 mM HEPES, 2 mM DTT and 20 mM Na2Mo04, pH 7.6 at 0 C) in a glass homogenizer with a Teflon pestle milled to a clearance between the pestle and homogenization tube of .125 mm on the radius (to minimize rupture of the brain cell nuclei (McEwen and Zigmond, 1972). The crude homogenate was centrifuged at 100,000 g for 20 min and the supernatant recentrifuged at 100,000 g for an additional 60 min to yield cytosol. During these centrifuge runs, and during all other procedures, unless otherwise indicated, careful attention was paid to maintaining the cytosol at 0-2 C. Final protein concentrations were typically in the 6-8 mg/ml

range. For experiments involving occupied (activated or unactivated) receptors, cytosol samples were incubated with 20 nM [3H]TA for 24 to 40 hours at 0 C with or without a 200-fold excess of unlabeled TA.

Experiments involving hydrophobic interaction chromatography of unoccupied receptors required that the individual fractions collected from the hydrophobic and control columns be postlabeled with 20 nM [3H]TA +/- 4 um [1H]TA. Prior to hydrophobic interaction chromatography of unlabeled receptors, cytosol was run on Sephadex G-25 columns equilibrated and eluted with the appropriate buffer to be used for subsequently eluting the hydrophobic columns.

Activation and Removal of Unbound Steroid

[3H]TA-labeled cytosol samples to be activated were first run on Sephadex G-25 columns (0.6 x 14 cm) equilibrated with buffer containing 20 mM HEPES and 2 mM DTT only. This column run resulted in the removal of both free steroid and molybdate, allowing for uninhibited activation (Luttge and Densmore, 1984; Luttge et al., 1984a,b,d). The bound fraction was then incubated at 22 C for variable periods of time to determine the minimum period of time required to achieve total activation. The degree of activation was determined by DNA-cellulose binding assay. For the purpose of hydrophobic interaction chromatography of activated receptors, the maximally activated receptor preparation was again run on a second Sephadex G-25 column equilibrated with the appropriate buffer (the same buffer used to equilibrate and elute the subsequent alkyl agarose columns). For unactivated receptors, the cytosol was treated in an identical fasion except that the first Sephadex G-25 column was equilibrated and eluted with HEPES buffer containing 20mM molybdate and 2 mM DTT and the bound fraction was

subjected to a 0 C (rather than a 22 C) incubation prior to running on the second Sephadex G-25 column (identical to the second column run for the activated preparation).

In the case of postlabeling of unoccupied receptor fractions or monitoring of dissociation occurring during the hydrophobic column runs of prelabeled receptors, all or part of each hydrophobic column fraction was again subjected to Sephadex G-25 chromatography to remove free steroid.

DNA-cellulose Binding Assay

Calf thymus DNA-cellulose, prepared originally by the method of Alberts and Herrick (1971), was added to HEPES buffer containing 2 mM DTT to yield a final concentration of 10 mg/ml (4.1 mg DNA/g DNA-cellulose). In a typical assay (run as duplicates) a 100 ul aliquot of [3H]TA- labeled cytosol was added to 300 ul of the DNA-cellulose slurry. The mixture was then vortexed gently and incubated at 0 C for 60 min. Assay tubes were oscillated at 150 rpm throughout the incubation and vortexed gently every 10 min. The DNA-cellulose was collected by centrifugation (2000 x g for 5 min), the supernatant discarded and the DNA-cellulose pellet washed 3 times with 1 ml of HEPES buffer plus 2 mM DTT. The entire pellet was resuspended in deionized water and transferred to scintillation vials for determination of bound radioactivity. DNA- cellulose was prepared in buffer not containing molybdate in order to reduce the concentration of molybdate during the binding assay, since high concentrations of molybdate were found to reduce the efficacy of receptor binding to DNA-cellulose (Luttge et al., 1984a).

Receptor Stability Determinations

Prelabeled receptor preparations (both activated and unactivated), were run on Sephadex G-25 columns equilibrated and eluted with 50 mM molybdate and varying concentrations of KCl (0 - 2,000 mM) in order to determine the effects of ionic strength on receptor stability. A fraction of the bound fraction was immediately run on a second Sephadex G-25 column prior to specific binding determination, while the remainder was incubated for 4 hr at 0 C prior to being run on a second Sephadex G-25 column and specific binding determined.

Results

The initial experiment in the investigation of glucocorticoid receptor hydrophobicity involved hydrophobic interaction chromatography of activated and unactivated glucocorticoid receptor complexes on a limited Shaltiel series using a series of elution buffers of decreasing hydrophobic character. This experiment used a series of hydrophobic gels created by covalently attaching alkyl groups of increasing chain length to agarose including propyl (n = 3 carbons), hexyl (n = 6), octyl (n = 8), decyl (n = 10) and dodecyl (n = 12) agarose, along with unmodified agarose as a control. Activated and unactivated receptor preparations equilibrated in 600 mM KCl and 50 mM molybdate were applied to each of the columns (containing 1 ml of gel) and then eluted step wise with the following buffers (each of which contained 50 mM molybdate in HEPES buffer): 600 mM KCl, 300 mM KCl, 0 mM KCl, 10% glycerol and 30% glycerol. Results (not shown) indicated that activated preparations were retained longer than unactivated and that this difference was the largest on the hexyl agarose columns. Both receptor forms appeared to be eluted completely with higher salt concentrations on all gels except

octyl, decyl and dodecyl which exhibited variable retention of both forms of the receptor in the presence of the more hydrophobic elution buffers.

Further refinement of the procedure to investigate receptor hydrophobicity next involved hydrophobic interaction chromatography of activated and unactivated glucocorticoid receptor complexes on a more complete Shaltiel series of alkyl agarose columns using a series of elution buffers of decreasing hydrophobic character. In addition to the original gels used, butyl (n=4), pentyl (n=5) and phenyl (n=P) agarose were added and the gel volume was increased to 1.5 ml. In addition, smaller fractions (0.5 ml) were collected to increase resolution and a different series of step wise buffer elutions were used including: 600 mM KCl, 0 mM KCl and 10% ethylene glycol (all in HEPES buffer plus 50 mM molybdate). A final elution of 6 M urea was used to remove those receptors (and other proteins) that were strongly retained throughout all of the elution steps. With the higher resolution resulting from smaller fraction size, it was obvious that activated receptors were retained longer than unactivated receptors run on butyl, pentyl and hexyl agarose columns. On all three columns, however, both forms of the receptor were eluted completely, though at different rates, by 600 mM KCl. Phenyl agarose strongly retained the activated receptor, whereas most of the unactivated form of the receptor was eluted rapidly under the high ionic strength conditions. Octyl, decyl and dodecyl agarose retained the receptors more than the other gels except that there were no significant differences between the activated and unactivated forms on these columns. Overall, the pentyl agarose exhibited the greatest differences in retention between the activated and unactivated forms

while still allowing for both forms to be eluted completely under the same buffer conditions.

Since the results of the previous experiments indicated that any binding retained by any of the hydrophobic columns after adequately washing with 600 mM KCl could not be eluted with anything tested short of denaturing solutions such as 6 M urea, hydrophobic interaction chromatography of activated and unactivated glucocorticoid receptor complexes on a Shaltiel series using only 600 mM KCl was performed. This experiment was similar to the previously described experiment except that only one elution buffer was used instead of a series of different buffers. The results basically replicated those of the previous experiment in that the activated form of the receptor consistantly shows a greater degree of retardation on most of the columns with pentyl, hexyl and phenyl agarose providing the greatest discrimination between the two forms (Figure 2-1).

Since high ionic strength elution buffers (HEPES buffer plus 50 mM molybdate and 600 mM KCl) were able to discriminate between the activated and unactivated forms of the receptor on virtually all of the columns tested while allowing all of the binding to be eluted, the effects of using lower or higher ionic strength buffers to equilibrate and elute the hydrophobic columns were investigated. Because of uncertainties regarding the activation of unactivated glucocorticoid receptors and the stability of glucocorticoid binding under high salt conditions, hydrophobic interaction chromatography of activated and unactivated glucocorticoid receptor complexes on a Shaltiel series equilibrated and eluted with only HEPES buffer and 50 mM molybdate (no KCl) was first attempted (Figure 2-2). Proply, butyl, pentyl and hexyl

Figure 2-1. Hydrophobic interaction chromatography of unactivated and activated Type II glucocorticoid receptor complexes on a Shaltiel series equilibrated and eluted with HEPES buffer containing 50 mM molybdate and 600 mM KCl. Brain cytosol prepared with HEPES buffer containing 20 mM molybdate and labeled with 20 nM [3H]TA for 40 hr at 0 C, was run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer plus either 0 or 20 mM molybdate to remove the molybdate and/or free steroid. The molybdate-free cytosol was activated by a 24 min incubation at 22 C (solid triangle), whereas the molybdate-containing, unactivated cytosol was left at 0 C (solid circles). Both groups were subsequently run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer containing 50 mM molybdate and 600 mM KCl prior to being run on each of the alkyl, phenyl and control agarose columns equilibrated and eluted with the same buffer. Cytosol (0.5 ml) was applied to the columns and 10 fractions (0.5 ml) were collected. Binding is expressed as percent of the total counts (> 15,500 cpm) applied to each column. Results presented here are representative of 3 independent replications. 0 = unmodified agarose (control), 3 = propyl agarose, 4 = butyl agarose, 5 = pentyl agarose, 6 = hexyl agarose, 8 = octyl agarose, 10 = decyl agarose and 12 = dodecyl agarose.

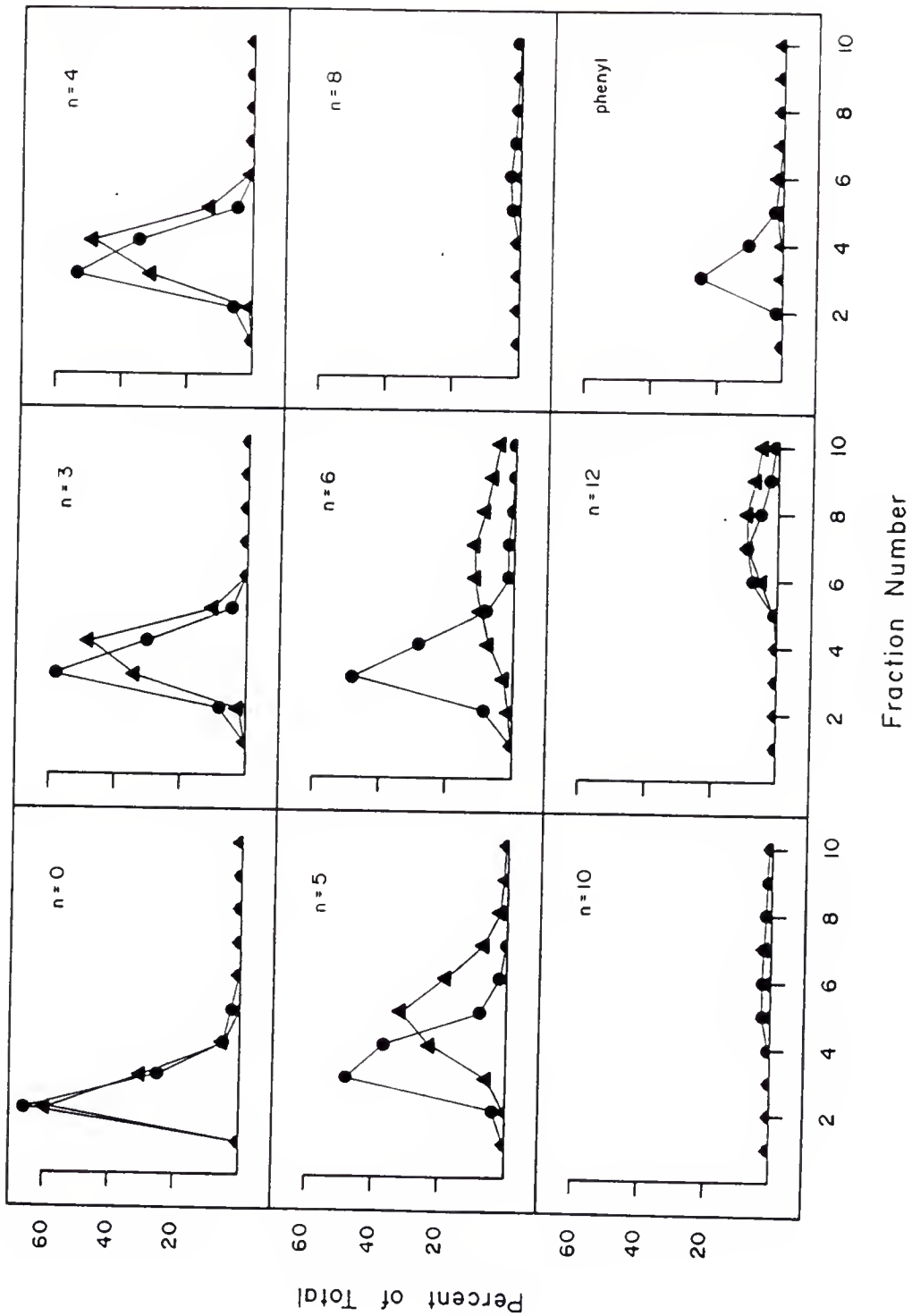
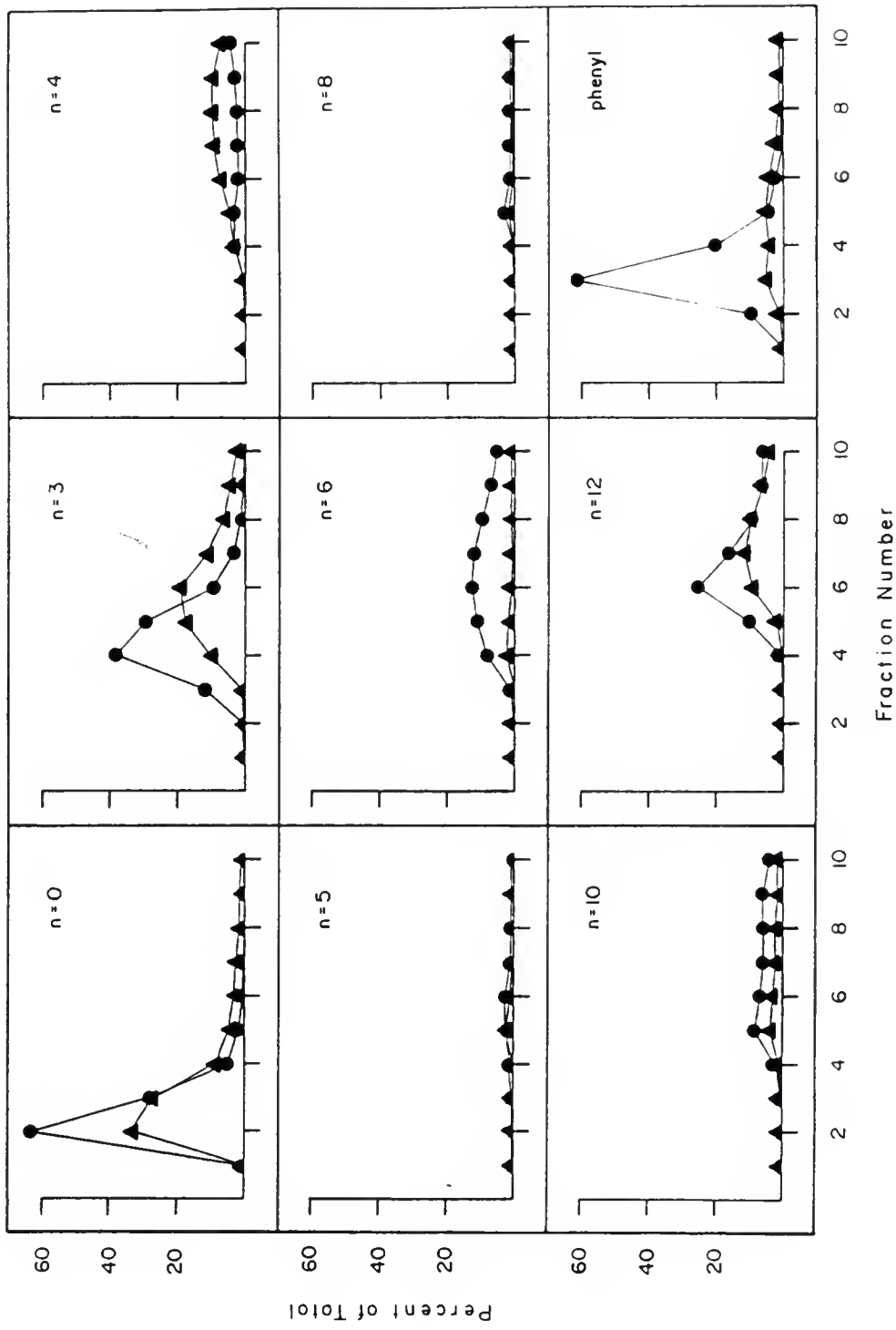

n = 0
n = 3
n = 4
n = 5
n = 6
n = 8
n = 10
n = 12
phenyl
60
40
20
2
4
6
8
10
Percent of Total
Fraction Number

Figure 2-2. Hydrophobic interaction chromatography of unactivated and activated Type II glucocorticoid receptor complexes on a Shaltiel series equilibrated and eluted under low salt conditions (HEPES buffer containing 50 mM molybdate and no KCl). Brain cytosol was prepared, labeled and activated (solid triangles) or left unactivated (solid circles) as described in Figure A. Both groups were subsequently run on Sephadex G-25 columns equilibrated and eluted with 50 mM molybdate, but no KCl, prior to being run on each of the alkyl, phenyl and control agarose columns equilibrated and eluted with the same buffer. Cytosol (0.5 ml) was applied to each column and 10 fractions (0.5 ml) were collected. Binding is expressed as percent of the total counts (> 15,000 cpm) applied to each column. Results presented here are represetative of 3 independent replications. 0 = unmodified agarose (control), 3 = propyl agarose, 4 = butyl agarose, 5 = pentyl agarose, 6 = hexyl agarose, 8 = octyl agarose, 10 = decyl agarose and 12 = dodecyl agarose.


n=0
n=3
n=4
n=5
n=6
n=8
n=10
n=12
phenyl
60
40
20
2
4
6
8
10
Percent of Total
Fraction Number

agaroses all showed an increased affinity for the unactivated receptor, resulting in varying degrees of increased retention when compared to elution by higher salt. Interestingly, pentyl agarose showed the greatest retention of the unoccupied receptor of any of the gels. In contrast, the unactivated receptor exhibited a decrease in retention on the phenyl agarose. There was no appreciable differences in the elution profiles obtained from octyl, decyl or dodecyl agarose in high or low salt. As was the case for unactivated receptors, propyl, butyl, pentyl and hexyl agaroses all showed an increased affinity for the activated receptor under low salt conditions, resulting in varying degrees of increased retention when compared to elution by higher salt. Interestingly, pentyl agarose exhibited the greatest retention of any of the gels examined for both the activated and the unactivated forms of the receptor. There was no appreciable differences in the elution profiles obtained from octyl, decyl, dodecyl or phenyl in high or low salt.

The finding that octyl agarose exhibited greater retention of both forms of the receptor than decyl agarose, which exhibited greater retention than dodecyl agarose regardless of ionic strength (one would have expected the reverse if these interactions were truly hydrophobic), led to a more extensive investigation of the interactions between the glucocorticoid receptor and these highly hydrophobic gels. Although free steroid is removed from the cytosolic preparations immediately prior to applying these preparations on the hydrophobic columns and binding with [3H]TA has been shown to exhibit high stability and very slow dissociation even under high salt conditions, the possibility that the hydrophobic alkyls might themselves be destabilizing the receptors via strong hydrophobic interactions had not been examined. This

involved replicating the previously described experiment whereby activated and unactivated receptors where applied to the complete Shaltiel series of columns equilibrated and eluted with HEPES buffer plus 50 mM molybdate and 600 mM KCl except that each fraction eluted from the column was divided into two equal halves. One half was counted directly as before while the other half was subjected to an additional bound/free separation on Sephadex G-25 columns. When the profiles determined by each of these methods for each of the gels were compared, there were no significant differences observed except in the case of the octyl, decyl and dodecyl agaroses. None of the counts eluting from these three columns eluted in the bound fraction during the subsequent Sephadex G-25 bound/free separation.

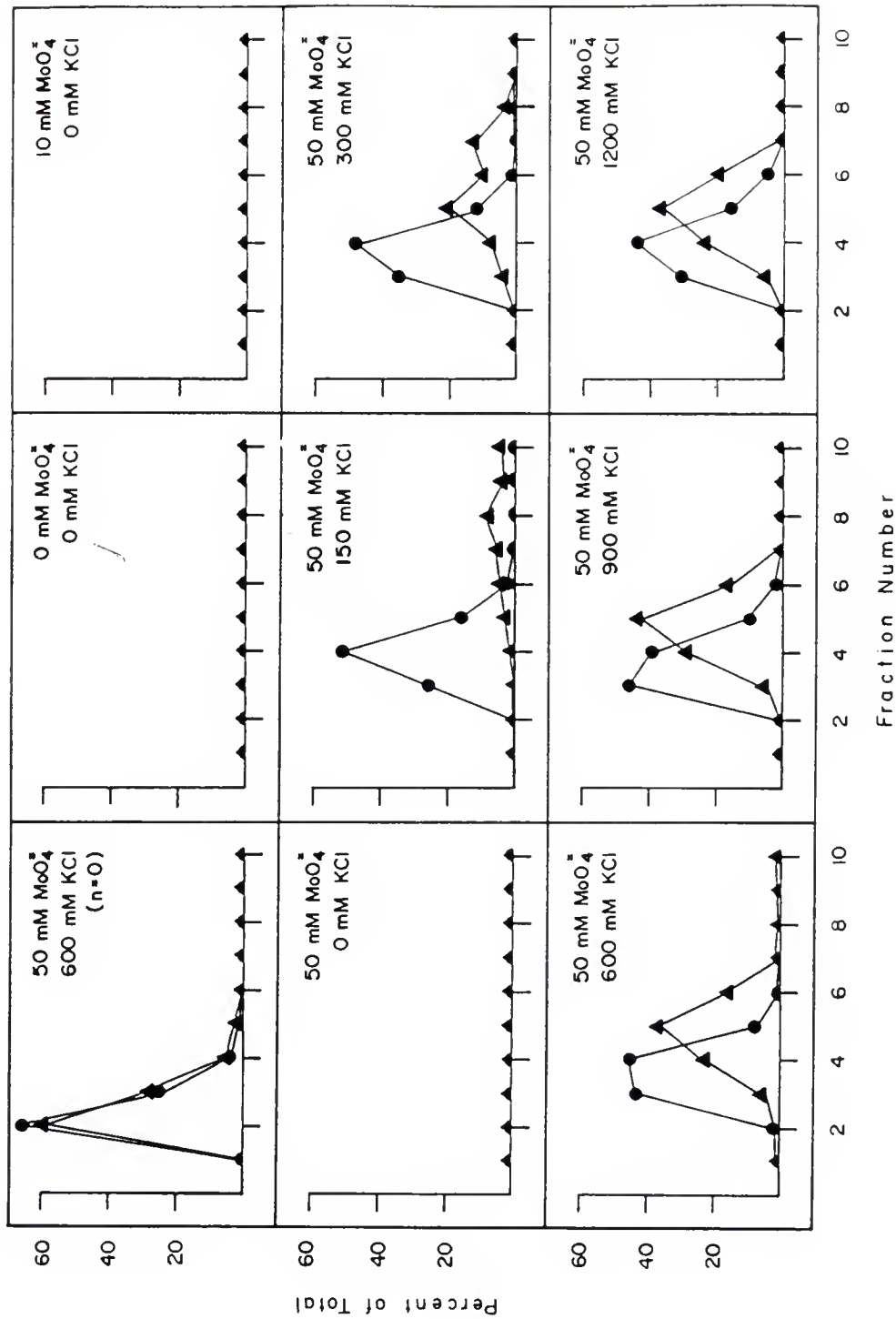
Since the results from the preceding experiment indicated how receptor instability and/or dissociation could complicate the interpretation of the results from hydrophobic interaction chromatography of these receptors, and since many of these experiments required higher concentrations of molybdate and potassium chloride than are generally encountered throughout most of the literature regarding glucocorticoid receptors, effects of these parameters on receptor binding properties obviously warranted further study. In this experiment, cytosol was prepared in the usual fashion in the presence of 20 mM molybdate and 2 mM DTT and labeled with 40 nM [3H]TA plus or minus unlabeled TA for 24 hours to achieve maximal binding. After this initial incubation, aliquots were taken to determine total and non-specific binding. Next, aliquots of prelabeled cytosol were applied to Sephadex G-25 columns preequilibrated with every possible combination of the following concentrations of sodium molybdate (0, 20 or 50 mM) and

KCl (0, 50, 150, 300, 600, 1200 or 1800 mM). Of the bound fraction collected from each of the 42 columns (21 total and 21 nonspecific), an aliquot was counted to represent the "zero hour" binding for each buffer condition. Each of the samples representing total binding was then further subdivided into two tubes, one containing 20 nM [3H]TA (for the determination of destabilization) and one containing 4 uM unlabeled TA (for the determination of destabilization plus dissociation). Each of the samples representing nonspecific binding was also subdivided into two tubes, one containing 20 nM [3H]TA and 4 uM unlabeled TA and the other containing only 4 uM unlabeled TA. After an 8 hour incubation at 0 C, aliquots of each of these experimental groups was again subjected to bound/free separation on Sephadex G-25 columns. Rates of destabilization and dissociation (which rarely exceeded a few percent) were very low for all groups, to the point that neither was likely to have been a significant factor in any of the hydrophobic interaction chromatography studies involving high concentrations of molybdate and/or KCl. It must be emphasized, however, that these results cannot necessarily be applied to the binding of this receptor to other ligands, such as DEX or corticosterone, or to the binding of this receptor under different temperature or pH conditions. However, since the conditions of this experiment matched very closely the conditions of the hydrophobic studies, any significant loss in binding seen during these studies is likely to be the result of destabilizing effects of the hydrophobic matrix instead of the buffer itself.

Since potassium chloride concentrations of up to 1200 mM with and without molybdate were shown to have little effect on the stability or rate of steroid (TA) dissociation of glucocorticoid receptors maintained

at low temperatures, and since retention of both activated and unactivated receptors was shown to increase dramatically on alkyl (particularly pentyl) agarose gels when ionic strength was lowered, the effects of ionic strength and molybdate concentration on hydrophobic interaction chromatography of both activated and unactivated receptors was investigated in greater detail. Unactivated receptor preparation was applied to pentyl agarose columns equilibrated in, and then eluted with, one of the following: HEPES only, 10 mM molybdate, 50 mM molybdate, 150 mM KCl and 50 mM molybdate, 300 mM KCl and 50 mM molybdate, 600 mM KCl and 50 mM molybdate, 900 mM KCl and 50 mM molybdate or 1,200 mM KCl and 50 mM molybdate (Figure 2-3). Despite the finding that high ionic strength had little effect on TA dissociation from glucocorticoid receptors, the eluted fractions were subjected to another bound/free separation step as an extra precaution. Pentyl agarose was chosen as the gel to study the effects of ionic strength since it was pentyl agarose that displayed total retention of both forms of the receptor under low salt conditions. Since the effect of molybdate (which had been present in all prior hydrophobic interaction chromatography experiments in this study) was unknown, the concentration of both molybdate and KCl were varied in this experiment. Columns eluted with HEPES only, 10 mM molybdate or 50 mM molybdate displayed total retention by the column. The elution profiles of all the KCl-containing buffers were very similar except that a very slight decrease in retention was observed going from 150 to 300 to 600 mM KCl. The differences between these three concentrations, though possibly significant, were not very dramatic. Elution profiles resulting from 600, 900 and 1,200 mM KCl showed virtually no changes. In a parallel

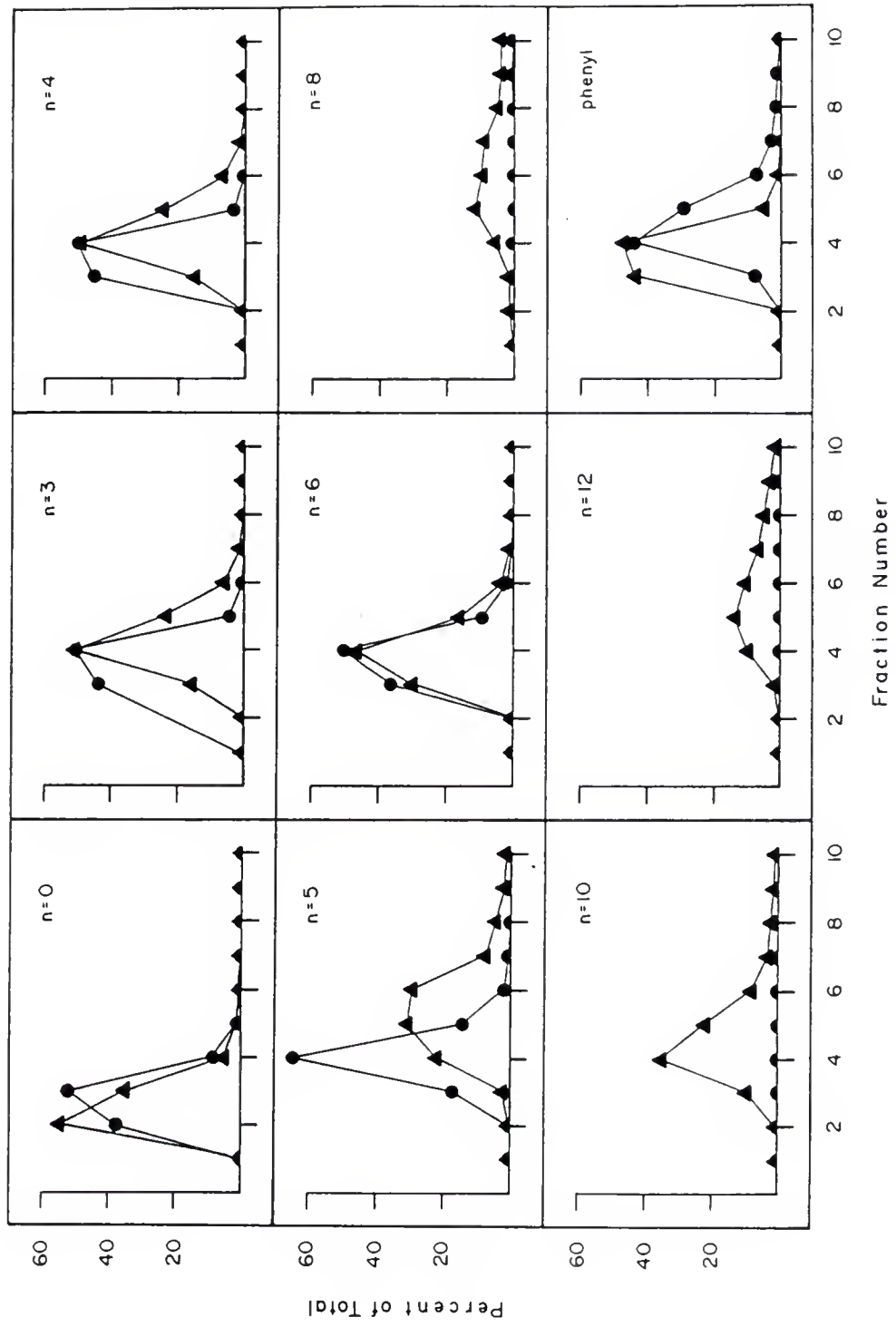
Figure 2-3. Hydrophobic interaction chromatography of unactivated and activated Type II glucocorticoid receptor complexes on pentyl agarose columns equilibrated and eluted with increasing ionic strength and molybdate concentrations. Brain cytosol was prepared, labeled and activated (solid triangles) or left unactivated (solid circles) as described in Figure 2-1. Both groups were subsequently run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer plus one of the following: no molybdate or KCl, 10 mM molybdate, 50 mM molybdate, 50 mM molybdate and 150 mM KCl, 50 mM molybdate and 300 mM KCl, 50 mM molybdate and 600 mM KCl, 50 mM molybdate and 900 mM KCl, 50 mM molybdate and 1200 mM KCl prior to being run on pentyl agarose columns equilibrated and eluted with the same buffer. Cytosol (0.5 ml) was applied to each column and 10 fractions (0.5 ml) were collected. Binding is expressed as percent of the total counts (> 13,700 cpm) applied to each column. Results presented here are representative of 3 independent replications. 0 = unmodified agarose (control) run only with 50 mM molybdate and 600 mM KCl, all other columns are pentyl agarose.


10 mM MoO4=
0 mM KCl
0 mM MoO4=
0 mM KCl
50 mM MoO4=
600 mM KCl
(n=0)
50 mM MoO4=
300 mM KCl
50 mM MoO4=
150 mM KCl
50 mM MoO4=
1200 mM KCl
50 mM MoO4=
900 mM KCl
50 mM MoO4=
0 mM KCl
50 mM MoO4=
600 mM KCl
Percent of Total
Fraction Number

experiment, an activated receptor preparation was run on identical columns under indentical conditions. Unlike the unactivated form, however, the activated form was also very strongly (but not completely) retained when eluted with 150 mM KCl and again (but to a slightly lesser degree) when eluted with 300 mM KCl. Elution profiles with 600, 900 and 1,200 mM KCl were all rather similar.

For the purpose of comparing the hydrophobic properties of the different forms of the glucocorticoid receptor to those of other well studied proteins, the next two experiments examined the hydrophobicity of two purified proteins, bovine serum albumin (BSA) and immuno-gamma-globulin (IgG). Each of the two proteins were first radiolabeled with [14C]formaldehyde (Rice and Means, 1971) and then run on a Shaltiel series using 600 mM KCl and 50 mM molybdate. All other conditions were identical to the previously described experiments for glucocorticoid receptors being run on a Shaltiel series under these same salt conditions. Like the unactivated glucocorticoid receptor, BSA exhibited some slight retention by the propyl, butyl, pentyl, hexyl and phenyl agaroses and was totally retained by the octyl, decyl and dodecyl gels (Figure 2-4). Like the unactivated receptor, BSA exhibited some slight retention by the propyl, butyl, pentyl, hexyl and phenyl agaroses and was totally retained by the octyl, decyl and dodecyl gels. The chromatographic behavior of IgG was significantly different from that of BSA on several of the gels in the series. Of particular interest were the differences between the two proteins on pentyl, octyl, decyl and dodecyl agaroses. The pentyl agarose exhibited a higher affinity for the IgG than the BSA although both proteins did elute from the column. The biggest difference occurred on the decyl agarose where the BSA was

Figure 2-4. Hydrophobic interaction chromatography of bovine serum albumen (solid circle) and immuno-gamma-globulin (solid triangle) on a Shaltiel series equilibrated and eluted with HEPES buffer containing 50 mM molybdate and 600 mM KCl. Both proteins were [14C]methylated (for detection) to low specific activity with [14C]formaldehyde and then diluted into HEPES buffer plus 50 mM molybdate and 600 mM KCl prior to being run on each of the alkyl, phenyl and control agarose columns equilibrated and eluted with the same buffer. Cytosol (0.5 ml) was applied to each columns and 10 fractions (0.5 ml) were collected. Results are expressed as percent of the total counts (> 20,000 cpm) applied to column. The profiles presented here are representative of 2 independent replications. 0 = unmodified agarose (control), 3 = propyl agarose, 4 = butyl agarose, 5 = pentyl agarose, 6 = hexyl agarose, 8 = octyl agarose, 10 = decyl agarose and 12 = dodecyl agarose.


n=0
n=3
n=4
n=5
n=6
n=8
n=10
n=12
phenyl
60
40
20
2
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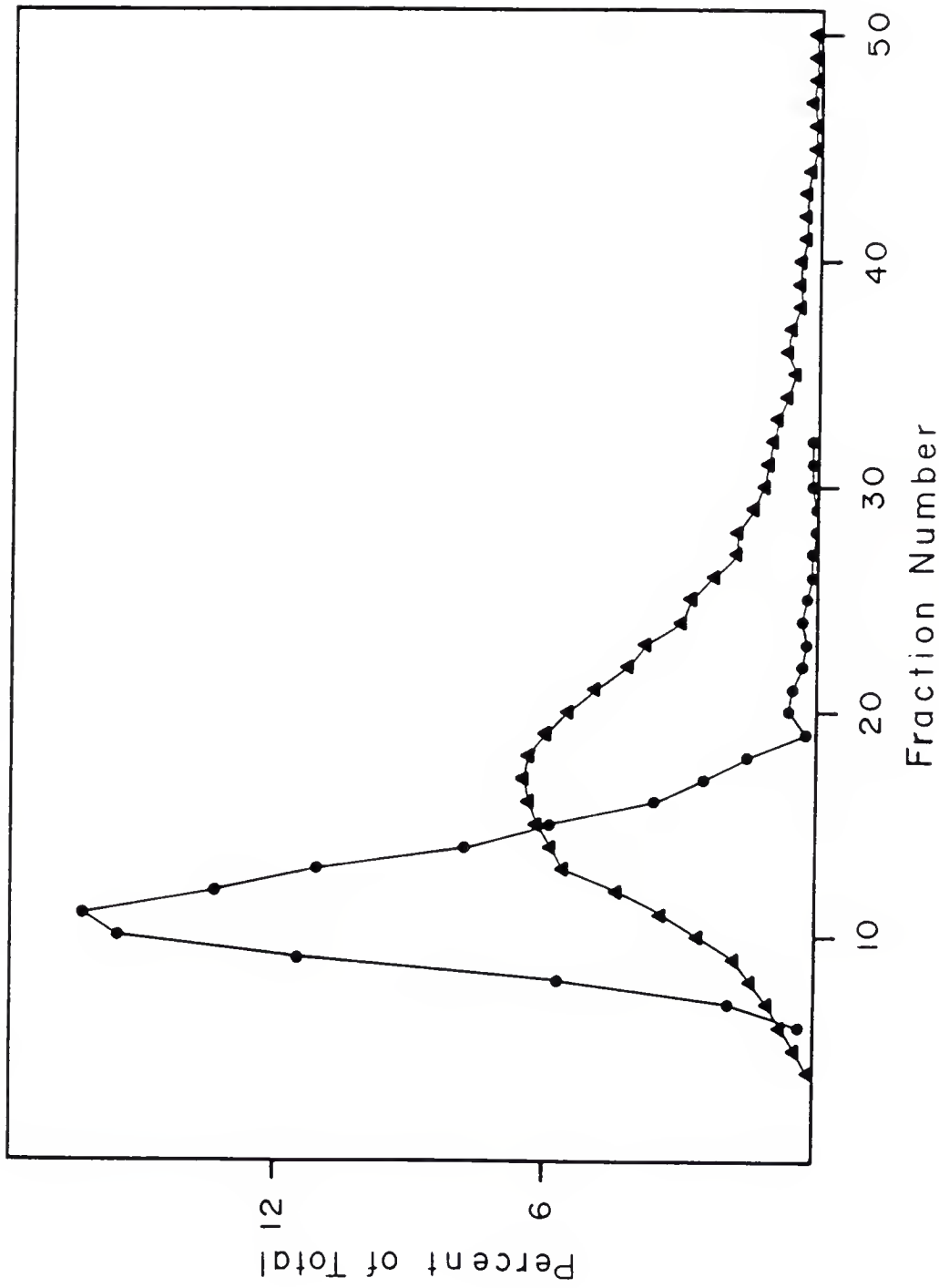
6
8
10
Percent of Total
Fraction Number

totally retained but the IgG eluted in a pattern similar to the propyl and butyl agarose profiles. Also of interest was the unexpected finding that IgG exhibited a greater affinity for hexyl agarose than for pentyl agarose and a greater affinity for decyl agarose than for octyl agarose. The effects of changing ionic strength on these profiles was not determined.

In an effort to improve resolution of the chromatographic profiles of the activated and unactivated forms of the glucocorticoid receptor as well as to increase separation between the 2 forms, these preparations were run individually on pentyl agarose columns much longer than those used for the experiments involving the complete Shaltiel series (7 ml as opposed to 1.5 ml). These preparations were run on columns equilibrated and eluted with 600 mM KCl and 50 mM molybdate and fractions of 0.5 ml were collected so that fraction size was smaller relative to gel volume, thereby increasing resolution further. As the results illustrate (Figure 2-5), the unactivated receptors were represented by the sharper, faster eluting peak, whereas the activated receptors were represented by a broader, more slowly eluting peak. Although a much high resolution was attained, the two different profiles didn't change much relative to one another when compared to their relative shapes and positions when run under identical buffer conditions on the smaller pentyl agarose columns. In other words, the separation between the two peaks was not significantly increased by increasing column length and there was still a significant amount of overlap between them. In an attempt to increase the separation between the two peaks, the experiment was repeated under identical conditions except that the KCl concentration was lowered from 600 mM to 400 mM since the affinity of activated receptors for pentyl

Figure 2-5. Hydrophobic interaction chromatography of activated and unactivated Type II glucocorticoid receptors on long (7 ml) pentyl agarose columns. Brain cytosol was prepared in HEPES buffer plus 20 mM molybdate and 2 mM DTT and incubated with 20 nM [3H]TA at 0 C for 24 hr. Aliquots were then either run on Sephadex G-25 columns equilibrated and eluted with 600 mM KCl and 50 mM molybdate (unactivated, solid circles) or run first on Sephadex G-25 columns equilibrated and eluted with only HEPES buffer, incubated at 22 C for 24 min to activate the receptor complexes and then run on a Sephadex G-25 column equilibrated and eluted with 600 mM KCl and 50 mM molybdate (activated, solid triangles). Aliquots (0.5 ml) of each treatment were run on pentyl agarose columns equilibrated and eluted with 600 mM KCl and 50 mM molybdate and 0.5 ml fractions were collected directly into scintillation vials for counting. Binding is expressed as percent of the total counts applied to the column eluting with each fraction. Each condition was run in triplicate and the profiles shown are representative.


Percent of Total
12
9
6
10
20
30
40
50
Fraction Number

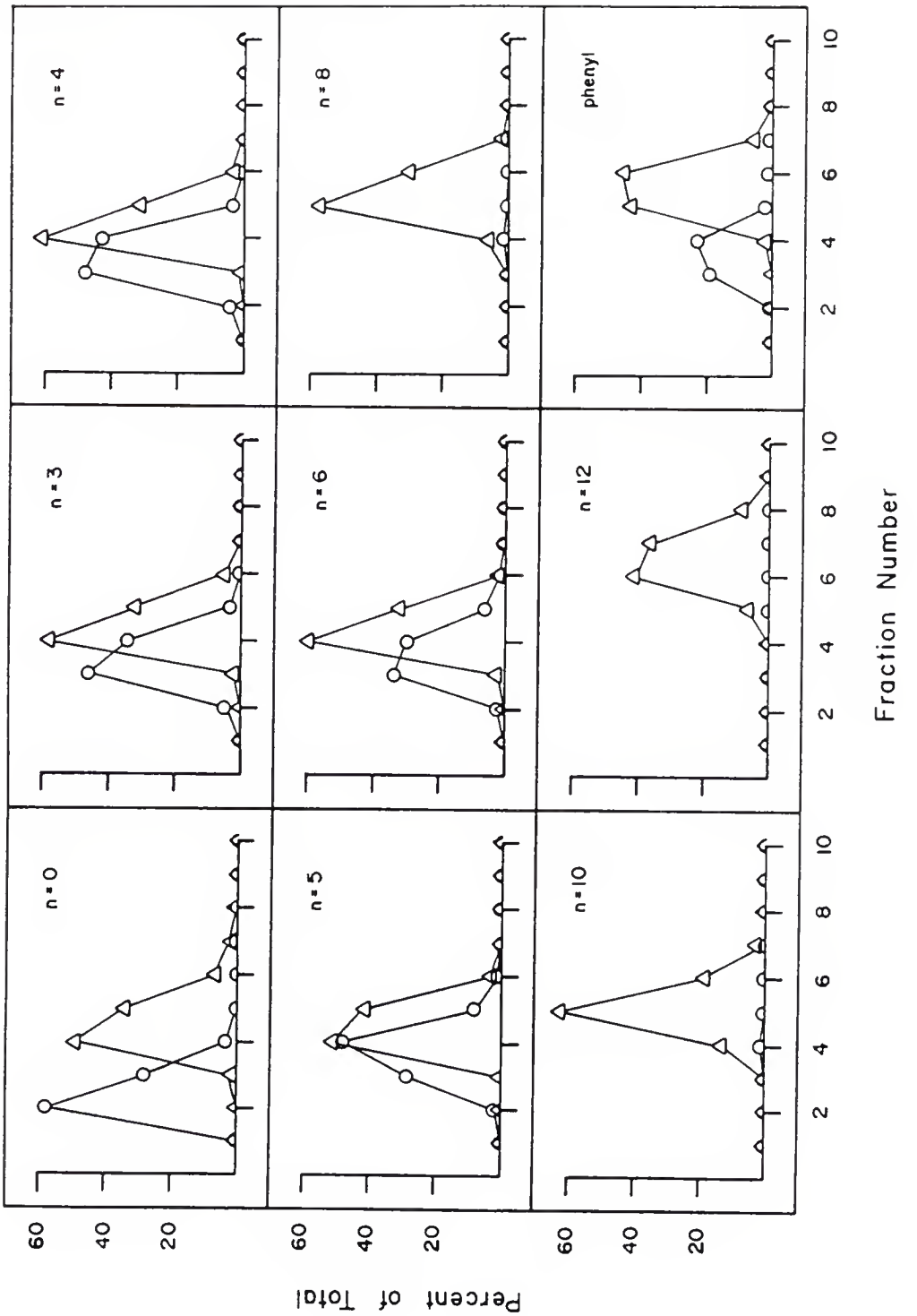
agarose had previously been shown to increase to a greater extent than the affinity of pentyl agarose for unactivated receptors when lowering the KCl concentration from 600 mM to 300 mM. The results were that under the 400 mM KCl elution conditions, the activated peak was broader than with 600 mM KCl elution while the unactivated receptor peak did not change significantly (data not shown). Overlap of peaks was still substantial.

To more closely investigate the relationship between column shape and elution profile on hydrophobic interaction chromatography columns, the next experiment examined the profiles for both the activated and unactivated forms of the glucocorticoid receptor on two dramatically different shaped pentyl agarose columns with the same gel volume. Although two different sizes of columns had been used previously, the shape difference (ratio of gel length to width) were not great and differences in gel volume, relative sample size and relative fraction size made comparisons of relative chromatographic resolution difficult. In this experiment, the gel volume for both columns was 7 ml, but the length to width ratio for one column was 18.6 (this column was identical in dimensions to the 7 ml column described in the previous experiment) while the length to width ratio for the second column, which was significantly shorter and rounder, was 0.5, but, as previously mentioned, contained an identical volumn of gel. There was therefore a 37.2-fold difference in the length to width ratio of the two columns. The size of the sample applied to each column as well as the size of the fractions collected from each column were identical and the elution buffer contained 600 mM KCl and 50 mM molybdate. The results of this experiment (data not shown) indicate that there were no major

differences in the overall chromatographic profiles (position of peak fraction, etc.) obtained from the two columns for either the activated or unactivated forms of the receptor although there appeared to be a very slight decrease in resolution (sharpness of peak) on the short wide column for the unactivated receptor. This slight decrease in resolution may have been related to technical problems in evenly applying such a small sample volume to such a large gel surface.

Since most of the work to date characterizing the glucocorticoid receptor has involved steroid labeled forms, little is known about the physicochemical properties of the unoccupied glucocorticoid receptor. Since hydrophobic interactions are thought to account for the high affinity binding of glucocorticoids to their receptors (Wolff et al., 1978; Alfsen, 1983; Eliard and Rousseau, 1984), the empty hydrophobic pocket exposed on a receptor whose binding site is unoccupied by a steroid ligand may have an effect on the overall hydrophobicity of the receptor that could have functional implications in vivo. This experiment examined the hydrophobicity of the unoccupied glucocorticoid receptor using a Shaltiel series of alkyl agarose and phenyl agarose columns under experimental conditions identical to those previously described except post-, instead of pre-, labeling was required (Figure 2-6) for analysis of activated and unactivated receptors when 600 mM KCl and 50 mM molybdate was used. This allowed for direct comparison of the results between all three forms of the receptor. Basically, the unoccupied receptor behaved almost exactly like the unactivated occupied receptor. Only slight differences between the two were detected on profiles from the pentyl agarose columns. To determine if the slight difference seen on this column was meaningful, unoccupied receptor

Figure 2-6. Hydrophobic interaction chromatography of unoccupied Type II glucocorticoid receptors and free [3H]TA on a Shaltiel series equilibrated and eluted with HEPES buffer containing 50 mM molybdate and 600 mM KCl. For unoccupied receptor runs (open circles), brain cytosol prepared in HEPES buffer plus 20 mM molybdate and 2 mM DTT was run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer plus 50 mM molybdate and 600 mM KCl prior to being run on each of the alkyl, phenyl and control agarose columns equilibrated and eluted with the same buffer. Cytosol (0.5 ml) was applied to each column and 10 fractions (0.5 ml) were collected and postlabeled with 20 nM [3H]triamcinolone acetonide for 40 hr at 0 C. For free steroid runs (open triangles), steroid was dried in a tube and resolubilized in HEPES buffer plus 50 mM molybdate and 600 mM KCl before 0.5 ml aliquots were applied to each column and fractions collected as above. Binding is expressed as percent of the total counts (> 14,050 cpm) applied to each column. Results presented here are representative of 3 independent replications. 0 = unmodified agarose (control), 3 = propyl agarose, 4 = butyl agarose, 5 = pentyl agarose, 6 = hexyl agarose, 8 = octyl agarose, 10 = decyl agarose and 12 = dodecyl agarose.


n=0
n=3
n=4
n=5
n=6
n=8
n=10
n=12
phenyl
60
40
20
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8
10
Percent of Total
Fraction Number

preparation was run on the long (7 ml) pentyl agarose column previously described for higher resolution analysis of the activated and unactivated forms. The chromatographic profile obtained for the unoccupied receptor turned out to be virtually identical to that obtained for the unactivated steroid-labeled form (data not shown).

The possibility of using hydrophobic interaction chromatography as a means of separating bound from free steroid for glucocorticoid receptor binding assays was investigated. The results of this experiment may also prove useful in interpreting data from experiments where some degree of dissociation was known to occur on the hydrophobic columns. This experiment examined the chromatographic profiles of free [3H]TA on a Shaltiel series of columns equilibrated and eluted with 600 mM KCl and 50 mM molybdate under conditions identical to those used for the hydrophobic interaction chromatography of various forms of the glucocorticoid receptor. Results indicate (Fig. 2-6) that the chromatographic profile of the free steroid on propyl, butyl, pentyl and hexyl agarose was identical to that for the free steroid on unmodified agarose. There was a slight increase in the affinity of [3H]TA for octyl and decyl agarose and an even greater retention by dodecyl and phenyl agarose. It should be noted that all of the steroid applied to each of the columns was eluted by the seventh or eighth fraction. When compared to the profiles for glucocorticoid receptors, the free steroid profile overlaped these profiles in virtually every case. When free steroid was run on a long 7 ml column identical to that previously described, it eluted about midway between the peaks for the unactivated and activated receptor and overlapped both, demonstrating that hydrophobic interaction chromatography under the conditions used in

these experiments is less suitable for bound/free steroid separations than chromatography on unmodified agarose.

Discussion

The results of this study indicate the presence of hydrophobic regions on the different forms of the glucocorticoid receptor in brain. This is in agreement with the findings of Lamb and Bullock (1983) for progesterone receptors in uterus and Bruchovsky et al. (1981) for androgen receptors in prostate. Even more important is the fact that the degree of surface hydrophobicity of the glucocorticoid receptor complex (assumed to be directly related to the degree of retention by the hydrophobic gels) appears to be dramatically increased upon heat activation of the receptor to the nuclear binding form. While such a change in the surface hydrophobicity of glucocorticoid receptor complexes associated with activation has been suggested by other workers reporting an increase in the partitioning of these complexes after activation into the less polar polyethylene glycol (PEG) layer in an aqueous dextran-PEG two phase system (Andreasen, 1978 and 1982; Andreasen and Mainwaring, 1980) and by this lab (Luttge et al., 1984d), which reported a large increase in the fractional adsorption of glucocorticoid receptor complexes to glass fiber (Whatman GF/C) filters following heat activation, hydrophobic interaction chromatography has never been used to study this phenomenon in glucocorticoid receptors. Furthermore, although it is possible that the dramatic increase in surface hydrophobicity associated with heat-induced activation could represent an artefact of the nonphysiological conditions existing during the in vitro activation process, this increased hydrophobicity is

nevertheless consistent with the presumed function of glucocorticoid receptors in general. It is certainly possible that the increased hydrophobicity of the activated receptor could account, at least in part, for the increased affinity for a number of different nuclear components including chromatin (Sakaue and Thompson, 1977; Simons et al., 1976), nucleosomes (Climent et al., 1977), DNA (Baxter et al., 1972; Rousseau et al., 1975; Sluyser, 1983), RNA (Tymoczko and Phillips, 1983), the nuclear matrix (Buttyan et al., 1983; Kirsch et al., 1986) and the nuclear membrane (Smith and von Holt, 1981). If the unactivated receptor resides in the cytosolic/cytoplasmic compartment, as originally thought (Szego, 1974), activation-induced increases in hydrophobicity might facilitate the permeation of the receptor through the nuclear membrane. If, on the other hand, the current controversial theory stating that unoccupied and occupied unactivated receptors actually reside in the nucleus in vivo (for review see Walters, 1985) is true, then increased hydrophobicity could decrease the likelyhood of "leakage" of activated receptors from nuclei by virtue of their greater affinity for nuclear components.

A Shaltiel series of alkyl agarose, as well as phenyl agarose, columns were used since the ideal gel(s) to study the surface hydrophobicity of the various receptor forms under different buffer conditions was unpredictable initially. Although it has been shown that the hydrophobicity of a linear aliphatic carbon increases linearly with carbon chain length (Tanford, 1972), it has been argued by others (Shanbhag and Axelsson, 1975), that the effective hydrophobicity depends also on the flexibility of the hydrocarbon chain and consequently on the degree of interaction within such chains, particularly for long chains.

The mechanisms by which proteins are adsorbed to hydrophobic matrices are no doubt complex and this problem can be exemplified in the present study by the case of the adsorption of IGG on a series of alkyl agarose columns, in which case a decrease in adsorption between pentyl and hexyl and between octyl and decyl agarose. This is similar to the case of the adsorption of erthrocytes on a series of alkyl agarose derivatives, wherein a decrease in adsorption between hexyl and octyl agarose was noticed to occur without an obvious explanation. The present study found that bovine serum albumen had a higher affinity for pentyl agarose than did IGG, whereas the reverse was true for octyl, decyl and dodecyl agaroses.

Another problem adding to the unpredictable nature of hydrophobic interactions stems from the fact that some hydrophobic ligands denature proteins through a "detergent-like" action (Hofstee, 1973). This was evidenced by the fact that what initially appeared to be a partial elution of both activated and unactivated receptors from octyl, decyl and dodecyl agaroses, later proved to be dissociated free steroid resulting from the denaturation of the receptors. The degree of denaturation increased with alkyl chain length with dodecyl agarose producing the greatest effect. In addition, the degree of denaturation for activated and unactivated forms appeared to be roughly equivalent, implying the possibility that the disruptive hydrophobic interaction leading to loss of steroid binding involved a region or regions common to both forms of the receptor. This finding may be relevant to the interpretation of other studies involving hydrophobic interaction chromatography. For instance, Lamb and Bullock (1983) reported that a large volume of buffer was required for elution of the progesterone

receptor from decyl agarose, which they claimed was probably due to slow equilibrium of the column with elution buffer. Another possibility is that the progesterone receptors tightly bound to the decyl agarose may have been undergoing a slow denaturation resulting in broad profiles of eluted radioactivity representing free steroid only. When dissociation was taken into account in the present study, no steroid receptor complexes could be eluted intact from octyl , decyl or dodecyl agarose using any of a number of buffers including some with increased "hydrophobic character" such as glycerol or ethylene glycol. Thurow and Geisen (1984) have reported that polypropylene glycol/polyethylene glycol block polymers prevent both the adsorption of dissolved proteins to hydrophobic interfaces and the resultant aggregation and denaturation. However, it is not known whether or not these polymers can successfully elute the intact receptors from highly hydrophobic interfaces if they are already attached and the presence of such polymers prior to any hydrophobic interactions would reduce or eliminate any such interactions, rendering the whole procedure useless.

Overall, the most promising results were obtained with the pentyl, hexyl and phenyl agarose gels. Phenyl-agarose has reportedly been effective in hydrophobic studies of the progesterone receptor (Logeat et al., 1981; Lamb and Bullock, 1983) and in differentiating between the activated and unactivated forms of the estrogen receptor (Gschwendt and Kittstein, 1980). The activated form of the glucocorticoid receptor bound more tightly to phenyl agarose than to alkyl agarose (excluding octyl or longer alkyl agaroses). When calculated as the change in standard free energy on transfer from an aqueous solution to a solution of pure hydrocarbon, a phenyl group is less hydrophobic than a pentyl

group (Rosengren et al., 1975; Lamb and Bullock, 1983). A portion of the unactivated receptor was retained by phenyl agarose under high salt conditions, while the same receptor preparation was only slightly retarded on the pentyl and hexyl agarose columns. While the activated form of the receptor eluted much more slowly from the pentyl and hexyl agaroses than did the unactivated form, the activated form was completely retained on the phenyl agarose under low and high salt conditions. Shaltiel (1974) has proposed that adsorption requires the interaction of an alkyl residue of sufficient length with a hydrophobic "pocket" within the protein. It has been suggested that the aromatic ring on phenyl agarose may be more complementary than alkyl groups to the contours of the hydrophobic "pocket" on the progesterone receptor (Lamb and Bullock, 1983). Because of similarities in amino acid sequence between the two receptor types (Conneely et al., 1986), such a pocket may also exist in or on the glucocorticoid receptor and may be made more accessible for hydrophobic interactions by heat activation. Alternatively, the receptor may form a charge transfer complex with the phenyl group, thus increasing the interaction. One might therefore conclude that phenyl agarose is the best gel for differentiating between the activated and unactivated forms of the glucocorticoid receptor based on differences in surface hydrophobicity. Unfortunately, the affinity of the activated receptor for this particular matrix prevents removal of the receptor from the matrix in an intact form. In addition, minor changes in hydrophobic interactions between the activated receptor and the matrix (i.e., as caused by changes in ionic strength, temp., etc.) cannot be readily monitored by phenyl agarose and therefore alkyl agaroses are best suited for such studies.

A number of factors in addition to the hydrophobicity of the attached groups influence the adsorption of receptors and other proteins to hydrophobic matrices. Jennissen (1976) has presented evidence in favor of the idea that the adsorption of proteins to alkyl agarose derivatives takes place at a critical alkyl group density. In other words, this hypothesis considers that the protein needs to present multiple attachment points in order to be adsorbed by a determinate member of the series of alkyl agarose derivatives. Another factor affecting the chromatographic behavior of a given protein on a particular hydrophobic matrix is the overall protein concentration applied to the column. Small amounts of protein bind more homogeneously on a particular column than a large amount. This should not have been a factor, however, in the differences in hydrophobicity exhibited by activated and unactivated receptors in this study since great care was taken to maintain the same protein concentration in all treatment groups and throughout all experiments. On the other hand, this could very well have been a factor in the chromatographic differences seen between the unpurified activated receptor and the purified activated receptor since there were hundreds- to thousands-fold differences in protein concentration (discussed in more detail in a later section). Another way in which protein concentration, especially in impure, crude preparations such as cytosol, could potentially affect the chromatographic profile of glucocorticoid receptors on these columns is through indirect, or secondary, interactions with other proteins bound tightly to the columns.

The influence of salt on the retention of receptors and other proteins to hydrophobic gels is probably due to a number of factors

acting on the protein as well as on the hydrophobic matrix. Pahlman et al. (1977) concluded that salting-out ions, such as potassium chloride, cause conformational, but not structural, changes in biomolecules. The "structure forming" properties of salting-out ions enhance intramolecular, as well as intermolecular, hydrophobic bonding as reflected by a stabilization of the hydrophobic core of the biomolecule (Hofstee, 1975). High concentrations of salting-out ions can adversely affect the solubility of a protein by decreasing the availability of water molecules in the bulk and increasing the surface tension of water, resulting in an inhancement of the hydrophobic interactions (Tanford, 1968; Melander and Horvath, 1977). It has been recently shown that the free energy of a protein is increased by addition of salting-out ions and this unfavorable free energy is smaller for the proteins bound to columns because of their smaller surface area exposed to solvent (Arakawa, 1986). The present study used, for the most part, a combination of two salts: sodium molybdate and potassium chloride. The 50 mM concentration of molybdate was required to prevent activation and to increase stability of all forms of the receptor under conditions requiring high concentrations of potassium chloride since lower concentrations of molybdate have been shown to be inadequate in preventing activation under high salt conditions (Weatherill and Bell, 1985). It was therefore not feasible to run meaningful experiments in the presence of potassium chloride only, although the possibility exists that the presence of molybdate may have influenced the effects that potassium chloride would have had (aside from increasing activation) on protein conformation and therefore, possibly, hydrophobic interactions. When the effects of salt concentration on the chromatographic profiles of

both activated and unactivated receptor complexes on pentyl agarose were examined, it was clear that all of the gels tested except phenyl agarose exhibited a greater affinity for both forms of the receptor under low salt conditions than under high salt conditions. Even the unmodified agarose exhibited a slight affinity for the activated form of the receptor under these conditions which may be due to low level electrostatic or other interactions with the gel matrix itself (Janson, 1967). Since this was opposite of what was expected, a more detailed study was carried out using only pentyl agarose and increasing concentrations of both molybdate and potassium chloride. Elution of activated receptors from the pentyl agarose required a higher concentration of potassium chloride than was required to elute the unactivated receptors. Although electrostatic interactions with the matrix (the alkyl groups are supposedly uncharged after attachment to an agarose matrix (Rosengren et al., 1975)) probably contributes to the higher affinity at lower salt concentrations, the effects observed are likely to involve a combination of electrostatic and hydrophobic interactions since the activated receptor elutes from ion-exchange columns at a lower salt concentration than the unactivated form (Sakaue and Thompson, 1977). Once the electrostatic interactions are overcome by using 600 mM concentrations of potassium chloride or greater, the chromatographic differences between the two forms are probably purely hydrophobic. It was reported by Peale et al. (1985) that the assayed levels of charged estrogen receptors, using both Sephadex gel filtration or dextran-coated charcoal treatments, in buffers containing 150-200 mM ionic strength (roughly physiologic) was approximately one half that of estrogen receptor levels assayed in buffers either at 0-50 or 400-450 mM

ionic strength. However, molybdate was shown to eliminate this reduction.

Although Wolf et al. (1978) have shown that the high affinity binding of a glucocorticoid by its receptor is largely due to hydrophobic interactions, the present study found little evidence that the primary site of the hydrophobic interactions with alkyl and phenyl agarose involved the steroid binding site. In fact, the chromatographic behavior of the unoccupied receptor differed little from that of the occupied unactivated receptor on practically all of the gels examined. These findings are in agreement with those of Lamb and Bullock (1983) who found that progesterone receptors bound to, and eluted from, alkyl agarose equally well in both the free and complexed form. This, in fact, might be expected in light of the high degree of specificity normally associated with steroid-receptor interactions. It has been implied, however, that steroid binding might be involved with some hydrophobic interactions between the rat estrogen receptor and Cibacron Blue (Tenenbaum and Leclerq, 1980).

In an attempt to improve the chromatographic resolution of the activated and unactivated glucocorticoid receptors eluting from alkyl agarose columns, pentyl agarose columns with various shapes (but with the same gel volume) were compared. In addition, elution flow rates were varied. None of these changes, however, resulted in any significant improvement in chromatographic resolution providing further support to the early observation by Frank and Evans (1945) that the hydrophobic interaction is a spontaneous process.

CHAPTER III

SULFHYDRYL REGULATION OF GLUCOCORTICOID BINDING CAPACITY

Introduction

The concentration of glucocorticoid receptors is subject to fluctuations resulting from several factors (De Nicola et al., 1982; Svec, 1985a), including autoregulation by the ligands themselves (Tornello et al., 1982; Ho-Kim et al., 1983; Muramatsu et al., 1983; Sapolsky et al., 1984; Svec, 1985b; Luttge et al., unpublished). The glucocorticoid-induced depletion of glucocorticoid receptors is reportedly due to an increased degradation of receptors and not due to a decreased rate of receptor synthesis (Svec and Rudis, 1981; McIntyre and Samuels, 1985). Androgens, on the other hand, have been associated with an augmentation of their receptors, apparently resulting from an increase both in receptor half-life and in rates of receptor synthesis (Syms et al., 1985). Glucocorticoid receptors may require an energy-dependent up-regulation or transformation before they are able to bind the steroid. Evidence for such an up-regulation in brain was presented by Turner and McEwen (1980), who reported a lack of depletion of hippocampal cytosol glucocorticoid receptors after multiple injections of [3H]corticosterone. These results could not be explained entirely by receptor synthesis and suggested that in the hippocampus an "excess" of glucocorticoid binding proteins exist, and that the availability of functional cytosol receptors may be regulated to maintain a relatively constant cellular level. Evidence of a rapid

down-regulation has also been observed. Recently, the loss of a biological response associated with such a down-regulation of glucocorticoid receptor levels was demonstrated in a mouse cell line (Danielsen and Stallcup, 1984). In addition to circulating levels of steroids, other factors may play a role in the regulation of steroid receptor concentration. Of particular relevance to the role of glucocorticoids in the nervous system is recent evidence that neural input influences significantly the levels of both androgen (Bernard et al., 1984) and glucocorticoid (DuBois and Almon, 1981) receptor levels in striated muscle as determined from denervation studies. Similarly, Cardinali et al. (1983) reported that pineal cytoplasmic and nuclear estrogen and androgen receptors are modulated by norepinephrine released from nerve endings at the pinealocyte level. These findings suggest that target cells may utilize a variety of homeostatic mechanisms to regulate the amount of receptor capable of interaction with the free steroid. Little is known, however, about these regulatory mechanisms and even less is known about the quantification, production and turnover of these cryptic (down—regulated) receptors. In addition, factors which stabilize or destabilize unoccupied receptors under in vitro conditions may also provide valuable information concerning the receptor and its regulation.

One potential mechanism of receptor up- and down-regulation for which there is fairly substantial evidence involves the reduction and oxidation of sulfhydryl groups on the receptor. Oxidation of receptor sulfhydryl groups (probably located in or near the steroid binding site) has been shown to reversibly inactivate glucocorticoid receptors in a number of tissues (Rees and Bell, 1975; Granberg and Ballard, 1977;

Sando et al., 1979; McBlain and Shyamala, 1980; Housley et al., 1982; Harrison et al., 1983; Densmore et al., 1984d; Izawa et al., 1984). These sulfhydryl groups are probably located in or near the steroid binding site as evidenced by the fact that mesylated derivatives of glucocorticoids have been shown to bind covalently to the receptor, presumably by means of an interaction between the mesylate group and a binding-site sulfhydryl group (Simons et al., 1980). The affinity labeling of the estrogen receptor has similarly indicated the presence of a thiol group at the binding site that appears to be directly involved in estradiol-receptor binding (Ikeda, 1982). Addition of sulfhydryl reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol leads to a total recovery of lost binding under appropriate conditions. Further evidence that the sulfhydryl-disulfide balance may be important in the regulation of glucocorticoid binding capacity was presented recently when it was shown that endogenous thioredoxin was responsible for up-regulation of glucocorticoid receptors in liver cytosol (Grippo et al., 1983). In addition to effects on binding capacity, oxidation of yet other sulfhydryl groups on the activated glucocorticoid-receptor complex by various sulfhydryl reactive agents has been shown to interfere with the DNA-binding ability of receptors from thymus (Bodwell et al., 1984) and most recently Kaufmann et al. (1986) has presented evidence suggesting that disulfide bonds mediate the association between the activated glucocorticoid receptor and the nuclear matrix, representing yet another possibility for the regulation of glucocorticoid action. Much work in this area remains to be done, however, before it can be determined whether these mechanisms are likely to be of significance in vivo.

Despite the growing body of evidence that ligand binding to the glucocorticoid receptor as well as binding of the activated glucocorticoid-receptor complex to nuclei or DNA are dependent upon the reduction of certain sulfhydryl groups on the receptor surface, there has been surprizingly little speculation concerning the possibility of thiol/disulfide exchange as a control mechanism for glucocorticoid action in vivo. The specific modulation of enzyme activity by post-translational modification of specific amino acid side chains is well established as a mechanism of metabolic regulation. Essentially all the amino acids possessing chemically reactive side groups are capable of undergoing some sort of posttranslational modification: the carboxyl groups of glutamate and aspartate, the imidazole side chain of histidine, the hydroxyl groups of serine, threonine and tyrosine, the amine group of lysine and even the guanidino group of arginine. However, the sulfhydryl group of cysteine is potentially the most reactive nucleophile in proteins. The process of thiol/disulfide exchange could provide a mechanism for the equilibration of the sulfhydryl oxidation state of proteins with the thiol/disulfide status of the surrounding environment. Although the regulation of enzyme activity by thiol/disulfide exchange was proposed some time ago (Barron, 1951), it has received less attention than other posttranslational modifications. According to Gilbert (1984), thiol/disulfide exchange as a biological control mechanism must exhibit at least the following six properties:

1. The intracellular thiol/disulfide status should vary in vivo in response to some metabolic signal. Isaacs and Binkley (1977a) reported that the disulfide-sulfhydryl ratios of subcellular (nuclear, microsomal

and cytosolic) fractions of rat hepatic tissue varied diurnally with the ratio lowest in the early morning (after feeding) and highest in the early evening (after fasting). The primary reaction was the reversible formation of mixed disulfides of glutathione with proteins and the change in the overall disulfide-sulfhydryl ratio was approximately 4-fold. These same workers (Isaacs and Binkley, 1977b) also reported changes in the disulfide-sulfhydryl ratio associated with cyclic AMP levels. Intaperitoneal injections of dibutyryl cyclic AMP induced an increase in hepatic glutathione protein mixed disulfides combined with a corresponding decrease in reduced glutathione and protein sulfhydryl. 2. Oxidation of protein sulfhydryl groups by thiol/disulfide exchange should activate some enzymes or receptors, inactivate others and not affect the activity of still others. Gilbert (1984) has compiled a large number of enzyme activities that have been suggested to be modulated by reversible thiol/disulfide exchange. Like some steroid receptors, many enzymes are reversibly inactivated by oxidation with a number of biologically occurring disulfides. One example is phosphofructokinase from either rabbit muscle (Gilbert, 1982) or mouse liver (Binkley and Richardson, 1982). Fructose-1,6-biphosphatase, however, undergoes a 2- to 4-fold activation in the presence of such disulfides (Gilbert, 1984).

Many enzymes have sulfydryl groups that can be chemically modified by a variety of sulfhydryl-specific reagents such as iodoacetamide, N-ethylmaleimide or Ellman's reagent with a resultant effect on enzyme activity. Most of these reagents are highly reactive toward sulfhydryl groups and somewhat less specific as a result. In addition, these reactive disulfides are much better oxidizing agents than most

biologically occurring disulfides. The enzymes aldolase and thiolase, for example, both have sulfhydryl groups that react with iodoacetamide, N-ethylmaleimide or reactive disulfides, however, incubation with 10mM glutathione disulfide at pH 8.0 for 24 hr (very harsh oxidizing conditions) had only a minimal effect on their activity (Gilbert, 1984). A similar situation has been observed with steroid receptors. Tashima et al. (1984) reported that monoiodoacetamide, N-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoate) all inactivated the binding capacity of unoccupied mineralocorticoid ([3H]aldosterone) receptors, while the binding capacity of these receptors was unaffected by endogenous oxidizing factors that lead to the temperature-dependent, reversible inactivation of glucocorticoid ([3H]dexamethasone) receptors under identical in vitro conditions (Emadian et al., 1985). Therefore, the presence of a chemically reactive sulfhydryl group is not in itself sufficient evidence to suggest regulation of any enzyme or receptor by thiol/disulfide exchange.

3. The thiol/disulfide redox potential of a regulated protein should be near the observed thiol/disulfide ratio in vivo. If the thiol/disulfide status of a regulated protein is in equilibrium with the thiol/disulfide status of the cell, the equilibrium constant for the thiol/disulfide exchange reaction

$$Ered + RSSR = Eox + RSH$$

$$Keq = [Eox][RSH]/[Ered][RSSR]$$

must lie near the intracellular thiol/disulfide ratio (Gilbert, 1984). According to this equilibrium model, changes in the ratio of oxidized to reduced receptor ([Eox]/[Ered]), and hence, the binding capacity, would result from changes in the thiol/disulfide ratio ([RSH]/[RSSR]). Such a

relationship has been observed, although the literature concerning the thiol/disulfide redox potentials of individual proteins is very limited. Two enzymes that do equilibrate with glutathione/glutathione-disulfide (GSH/GSSG) redox buffers in vitro are rabbit muscle phosphofructokinase (Gilbert, 1982) and chicken liver fatty acid synthetase (Walters and Gilbert, 1984). Unfortunately, there are no reports concerning redox potential measurement in steroid receptor systems. However, intracellular ratios of GSH/GSSG, the most predominant low molecular weight thiol/disulfide pair, typically decrease during in vitro procedures if precautions are not taken to "trap" thiols and disulfides at their original in vivo levels. This is because, at neutral pH, GSH is oxidized in air to GSSG; and due to the large excess of GSH over GSSG, postmortem oxidation of a small amount of the total GSH will result in a large artifactual increase in the observed GSSG concentration. One study reported an increase in GSSG concentration of about 50% in 100 min in acid-soluble extracts of rat liver adjusted to pH 7.4 (Vina et al., 1978). Such an increase would lead to changes in the GSH/GSSG ratio that would still be within the range of change observed to occur diurnally in rats (Isaacs and Binkley, 1977), indicating that changes seen in receptor binding activity due to postmortem oxidation of intracellular thiols might possibly reflect a receptor thiol/disulfide redox potential reasonably near the physiologically significant range. However, much work remains to be done before such a question can be answered.

4. Thiol/disulfide exchange reactions must be kinetically competent under physiological conditions. In order for the thiol/disulfide exchange between protein sulfhydryl groups and biological disulfides to

be considered a viable regulatory mechanism, the rate of this process in vivo must be at least as fast as the changes in the intracellular thiol/disulfide ratio. With some exceptions, most enzymes thus far studied would probably require catalysis of in vivo thiol/disulfide exchange in order for the process to be considered a viable mechanism of regulation (Gilbert, 1984). Since precise rates of thiol/disulfide exchange for steroid receptors have not been determined under defined conditions, it is currently unknown whether such a system would be kinetically competent in the absence of catalysis. However, recent reports have provided direct evidence for the existence of an endogenous thioredoxin-mediated reducing system in rat liver cytosol that maintains the glucocorticoid receptor in a reduced state in vitro in the presence of NADPH (Grippo et al., 1983, 1985). As for kinetic competence of this reaction, it should be noted that E. coli thioredoxin-(SH)2 reduces bovine insulin at least 10,000 times more rapidly than dithiotreitol at pH 7 (Holmgren, 1979). Endogenous mammalian thioredoxin activity has probably been undetected in many glucocorticoid receptor studies because of its easy inactivation by oxidation of structural SH groups (Luthman and Holmgren, 1982). Therefore, the same in vitro conditions (oxidation of reactive thiols) that inactivate unoccupied glucocorticoid receptors also simultaneously inactivate the enzymes that catalyze rapid reduction of receptor disulfides. The possible role of other enzymes that catalyze the reverse reaction (disulfide formation) have not been investigated with regards to steroid receptor systems. One such possibility, protein disulfide-isomerase, has been shown to have a rather broad substrate specificity, is widely distributed and has been detected in most vertebrate tissues (Hillson et al., 1984).

5. For regulated proteins, the oxidized and reduced forms should both be observable in vivo. The relative levels of the oxidized and reduced forms must change in response to changes in the cellular thiol/disulfide ratio. Although there is evidence that some enzymes, such as phosphofructokinase, exist in vivo in both an oxidized and reduced form, it is currently unknown whether both forms of the glucocorticoid, or any other steroid, receptor exist in vivo. This is for several reasons. First, in vitro preparation of cytosol for steroid receptor binding studies is not an instantaneous process and oxidation, as stated earlier, is most likely occurring if sulfhydryl group reducing agents are not present. However, in the presence of such reducing agents (such as dithiothreitol or 2-mercaptoethanol), the overall thiol/disulfide ratio of the cytosol is being shifted in favor of thiol formation. Even though nonenzymatic reduction of disulfides is relatively slow, dithiothreitol and other exogenously added reductants may reactivating or "up-regulating" enzymes such as thioredoxin that rapidly catalyze dithiol-disulfide oxidoreductions (Holmgren, 1977, 1979). These problems are magnified by the fact that, like cytosol preparation, the measurement of steroid binding capacity itself is very time consuming. At saturating concentrations of DEX or TA, low temperature incubations of 24 hours or longer are often required to reach equilibrium (or near equilibrium) binding. Finally, ratios of oxidized versus reduced receptors can not be assumed from receptor binding studies alone. The possibility exists that the oxidation or reduction of nonreceptor cytosolic components may indirectly modulate receptor binding capacity via nonthiol/disulfide exchange mechanisms.

6. The response of particular enzyme or receptor activities to oxidation by thiol/disulfide exchange should be consistent with the metabolic function of the enzyme or receptor. If changes in the thiol/disulfide ratio are coupled to changes in the activities of some regulated enzymes and receptors, and if one assumes that such ratios are based entirely on the feeding state of the animal (fasting leads to a decrease in the ratio (Isaacs and Binkley, 1977a)), three general consequences might follow (Gilbert, 1984). (1) Enzymes or receptors that should be active only in the fed state should lose activity on oxidation or gain activity on reduction. (2) Enzymes or receptors that should be active only in the fasted state should either gain activity on oxidation or lose activity on reduction. (3) Enzymes or receptors that must function in both the fed and fasted state should either not undergo thiol/disulfide exchange or the thiol/disulfide exchange should not affect the enzyme activity. Although, as stated previously, it has been reported that a decrease in the cellular thiol/disulfide ratio can be induced by starvation or the administration of agents that increase the intracellular concentration of cAMP, such as glucagon or epinephrine (Isaacs and Binkley, 1977a,b), the effects of many other factors (disease, stress, other hormonal systems) on cellular thiol/disulfide ratios simply has not been investigated in great detail. Therefore, while the relationship between some enzymes and changes in the cellular thiol/disulfide ratio might appear to be consistent with the metabolic function of the enzyme, the apparent absence or inconsistency of such a relationship based merely on the feeding status of an animal should not be taken as an absolute disqualification of redox control of a receptor system whose functions are as vastly complex as that of the

glucocorticoid receptor. In addition, tissue differences in cellular thiol/disulfide ratio, as well as levels of enzymes catalyzing thiol/disulfide exchange, are known to exist. This may, in part, explain the tissue differences reported in the dithiothreitol-inhibited instability of unoccupied glucocorticoid receptors (Granberg and Ballard, 1977), and may indicate tissue differences in the degree of redox control.

Materials and Methods

Chemicals, Steroids and Isotopes

[6,7-3H]Triamcinolone acetonide, or 9a-fluoro-11b,16a,17a,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide, ([3H]TA, specific activity = 37 Ci/mmol) and [6,7-3H]dexamethasone, or 9a-fluoro-16a-methylprednisolone ([3H]DEX, specific activity = 44.1-48.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Sephadex G-25 (fine) and Dextran T70 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Dinitrobenzoic acid (DTNB), N-ethylmaleimide NEM), iodoacetamide, iodoacetic acid, cystamine, glutathione disulfide (GSSG) and para-chloro-mercurisulfonate (PCMS), sodium-m-periodate, sodium-p-periodate, 6,6'-dithionicotinic acid (DTNT), methyl methane thiosulfonate (MMTS), mercuric chloride, sodium molybdate (Na2MoO4), calf thymus DNA-cellulose, glycerol, sucrose, activated charcoal, PPO (2,5-diphenyl-oxazole) and dimethyl POPOP (1,4-bis[2(4-methyl-5-phenyloxazoyl)]-benzene) and pentyl agarose were all purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were courtesy of Research Organics (Cleveland, OH). Scinti Verse II was

purchased from Fisher, Inc. (Fair Lawn, NJ). All other chemicals and solvents were reagent grade.

Animals

All studies used female CD-1 mice (Charles River Laboratories, Wilmington, MA) that were subjected to combined ovariectomy and adrenalectomy approximately 1 week prior to each experiment in order to remove known sources of endogenous steroids. Both operations were performed bilaterally via a lateral, subcostal approach under barbiturate anesthesia, and mice were given 0.9% NaCl (w:v) in place of drinking water. On the day of the experiment mice were anesthetized with ether and perfused slowly through the heart with ice-cold HEPES-buffered saline (20-30 ml, isotonic, pH 7.6).

Cytosol Preparation and Steroid Binding

Brains were removed from the perfused animals and homogenized (2x10 strokes at 1000 rpm) in 4 volumes of ice cold buffer containing 20 mM HEPES and 20 mM Na2MoO4, pH 7.6 at 0 C) in a glass homogenizer with a Teflon pestle milled to a clearance between the pestle and homogenization tube of 0.125 mm on the radius (to minimize rupture of the brain cell nuclei (McEwen and Zigmond, 1972)). The crude homogenate was centrifuged at 100,000 g for 20 min and the supernatant recentrifuged at 100,000 g for an additional 60 min to yield cytosol. During these centrifuge runs, and during all other procedures, unless otherwise indicated, careful attention was paid to maintaining the cytosol at 0-2 C. Final protein concentrations were typically in the 6-8 mg/ml range. After the appropriate experimental manipulations, cytosol samples were incubated with 20 nM [3H]TA or [3H]DEX for 24 to 40 hours at 0 C with or without a 200-fold excess of unlabeled steroid.

Charcoal Pretreatment

The dextran-coated charcoal (DCC) mixture was prepared overnight by adding 1.25% (w/v) activated charcoal and 0.625% dextran T70 to buffers identical in composition to that in which cytosol was prepared. Just prior to use, the desired volume of DCC suspension was centrifuged at 2,230 g for 10 min, the supernatant discarded and an equivalent volume of cytosol added to the pellet. The DCC was resuspended by gentle vortexing and the mixture incubated for 20 min at 0 C with 30 sec intermittent vortexing every 5 min. At the end of the incubation period, the dispersed DCC was pelletted by centrifugation at 2,230 g for 10 min and the resulting supernatant (i.e. the DCC-pretreated cytosol) carefully aspirated for subsequent steps. Control groups underwent identical procedural steps (in the absence of DCC) in parallel with their DCC-treated counterparts. The thermal stability of unoccupied receptors in each of these groups was tested by incubating the cytosol in a water bath maintained at the desired temperature for the period of time designated for each experiment. Following this "aging", the cytosol preparations were returned immediately to an ice bath. The protein concentration in such preparations ranged from 3.1 to 3.9 mg/ml (Lowry et al., 1951).

Hydrophobic Interaction Chromatography

Unlabeled cytosol was either up- or down-regulated by an appropriate incubation in the presence or absence of DTT, respectively (described in greater detail later). The different cytosolic preparations were then run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer containing 600 mM KCl and 50 mM molybdate at pH 7.6. Of the macromolecular fraction collected from these columns, 0.5 ml

was then run on a 7 ml pentyl agarose column equilibrated and eluted with HEPES buffer containing 600 mM KCl and 50 mM molybdate at pH 7.6. Fractions (0.5 ml) were collected and incubated with 20 nM [3H]DEX +/- 4 uM [1H]DEX for 40 hr at 0 C. The fractions were then assayed for specific binding.

Sucrose Density Gradient Sedimentation

Unlabeled cytosol was either up- or down-regulated by an appropriate incubation in the presence or absence of DTT, respectively. Aliquots (400 ul) were layered onto linear 5-20% sucrose density gradients (5 ml; prepared with HEPES buffer containing 20 mM molybdate and either with (for up-regulated receptors) or without (for down-regulated receptors) 2 mM DTT and centrifuged at 0 C for 19-20 hr at 200-234,000 g (average) in a Beckman SW 50.1 rotor. The cellulose nitrate tubes were punctured and 28-30 fractions (180 ul) collected and incubated with 20 nM [3H]DEX +/- 4 uM [1H]DEX in the presence of 2 mM DTT at 0 C for 24 hr. The individual fractions were then assayed for specific binding. Sedimentation coefficients (S20,w) were calculated from the linear regression of S20,w vs sedimentation distance (Martin and Ames, 1961) for the following standard proteins run in parrallel tubes: chicken ovalbumin (OVALB, 3.6 S), bovine serum albumin (BSA, 4.3 S), bovine gamma globulin (IgG, 7.4 S) and catalase (CAT, 11.3 S). The standard proteins were [14C]methylated (for detection) to low specific activity with [14C]formaldehyde by the method of Rice and Means (1971).

Steroid Binding Determination

In all experiments, bound [3H]-steroid was separated from free on Sephadex G-25 columns (0.6 x 14 cm) pre-equilibrated in buffer identical to that in which the cytosol was prepared. Duplicate aliquots from each

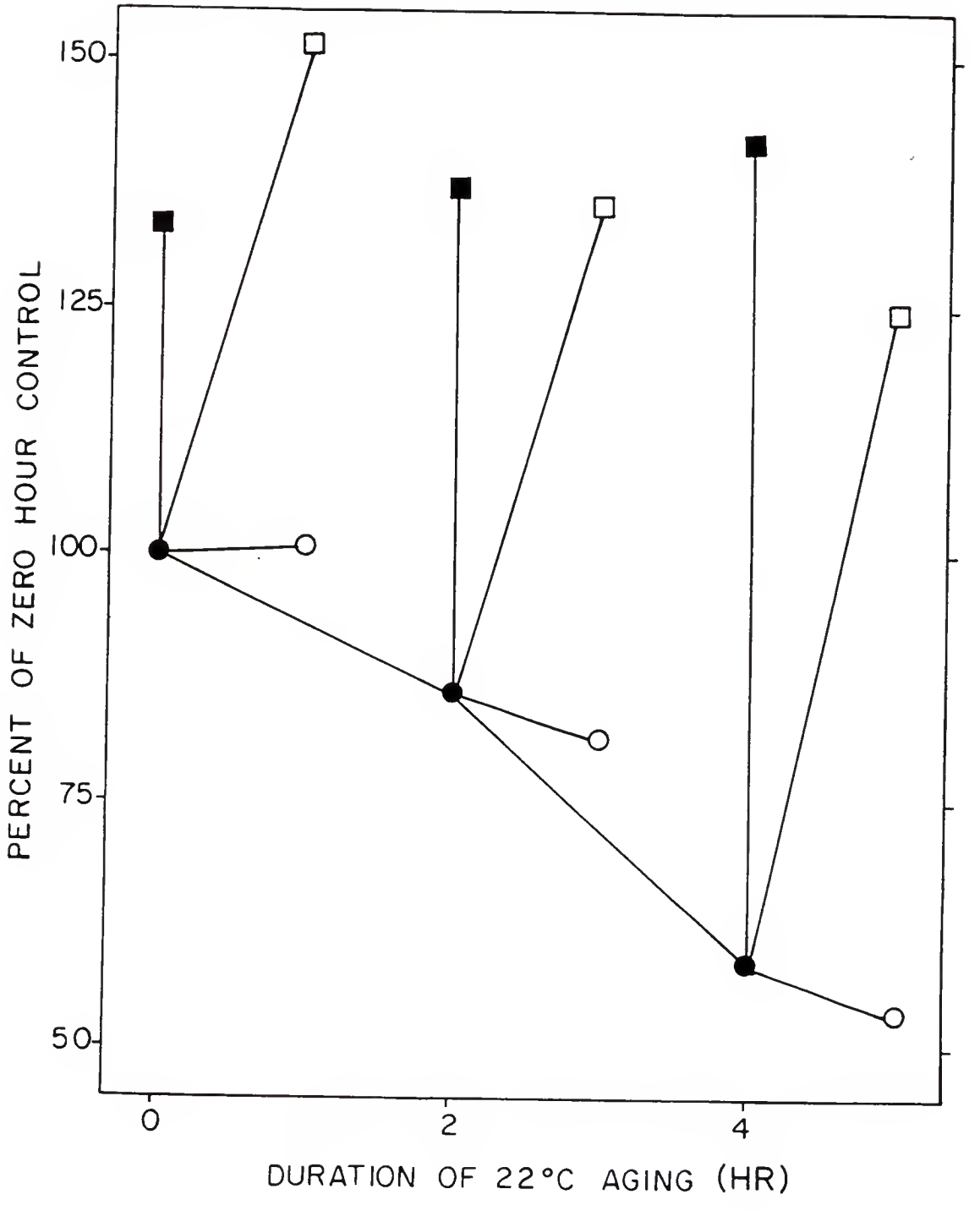
assay tube (BT and BNS) were layered onto separate columns, allowed to penetrate the gel and the macromolecular (bound) fraction eluted (with homologous buffer) directly into scintillation vials for liquid scintillation spectrometry.

Results

The purpose of this investigation was to determine the role of sulfhydryl oxidation-reduction in the regulation of steroid binding capacity of the unoccupied glucocorticoid receptor. It was therefore of importance to first characterize the time- and temperature-dependent changes in binding capacity occurring in cytosol in the absence of exogenously-added sulfhydryl reducing or oxidizing reagents. The first experiment investigated the temperature-dependent reversible loss of glucocorticoid binding capacity in the presence of molybdate, but in the absence of sulfhydryl reducing reagents such as DTT. Unlabeled cytosol was preincubated or "aged" at 22 C for 0, 2 and 4 hr, followed by addition of buffer with or without DTT prior to the steroid incubation. The experiment also examined the effects of aging of cytosol after the addition of DTT. Results (Figure 3-1) indicate that the binding capacity lost at 22 C in the presence of molybdate is completely restorable upon addition of DTT. Aging of the unlabeled cytosol after DTT addition had little effect on the binding capacity. Under these conditions, half of the maximal binding capacity (that measured after DTT addition) was reversibly inactivated after less than 4 hr of 22 C aging, although approximately 25% of the maximal binding capacity appeared to have already been lost prior to any 22 C aging whatsoever.

The next experiment was designed to more carefully investigate the phenomenon studied in the previous experiment, except the period of

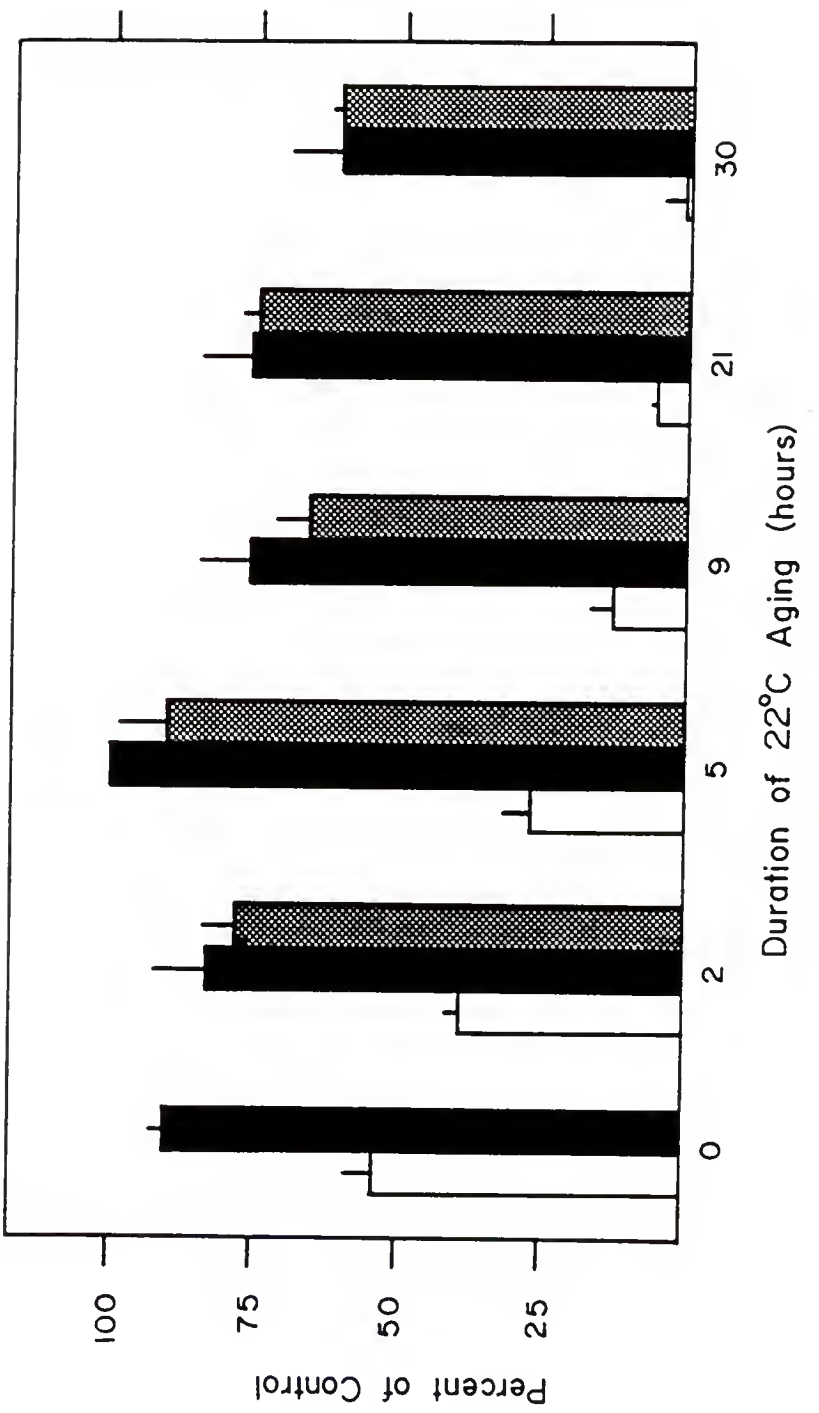
Figure 3-1. Temperature-dependent reversible loss of glucocorticoid binding capacity of unoccupied Type II glucocorticoid receptors in the presence of molybdate and absence of DTT. Solid circles represent brain cytosol prepared in HEPES buffer containing 20 mM molybdate that has been incubated at 22 C in the absence of steroid prior to a 24 hr incubation with 20 nM [3H]DEX +/- 4 uM [1H]DEX at 0 C. Solid squares represent the addition of DTT (2 mM final concentration) to the aged cytosol just prior to the initiation of steroid incubations. Open symbols represent one additional hr of 22 C aging after the addition of HEPES buffer containing either 20 mM molybdate only (open circle) or 20 mM molybdate plus 20 mM DTT (open squares, 2 mM final DTT concentration), but prior to steroid incubations. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the 0 hr control group (250 fmole/mg cytosol protein).


150
125
100
75
50
PERCENT OF ZERO HOUR CONTROL
0
2
4
DURATION OF 22°C AGING (HR)

preincubation aging was greatly extended to determine if the reversible down-regulation of glucocorticoid binding capacity could reach 100% under in vitro conditions. This experiment also sought to determine if unlabeled glucocorticoid receptors in the sulfhydryl down-regulated state were more or less stable for extended periods at 22 C than up-regulated unlabeled receptors. Like the previous experiment, unlabeled cytosol prepared with 20 mM molybdate was subjected to a preincubation aging at 22 C, but this time for 0, 2, 5, 9, 21 and 30 hr (Figure 3-2). In addition to the nonDTT-containing groups that received HEPES buffer with or without DTT after the 22 C aging, another group was included that contained DTT throughout the aging step. As in the previous experiment, suboptimal binding was encountered even prior to 22 C aging when DTT was absent from the cytosol. Down regulation appeared to reach virtual completion at 22 C, but preincubation on the order of 30 hr was required. Maximum binding capacity (i.e., after addition of DTT) diminished only slightly (about 30%) during the 30 hr period. There were clearly no significant differences in binding between the groups provided with DTT after the preincubation and those provided with DTT prior to the preincubation.

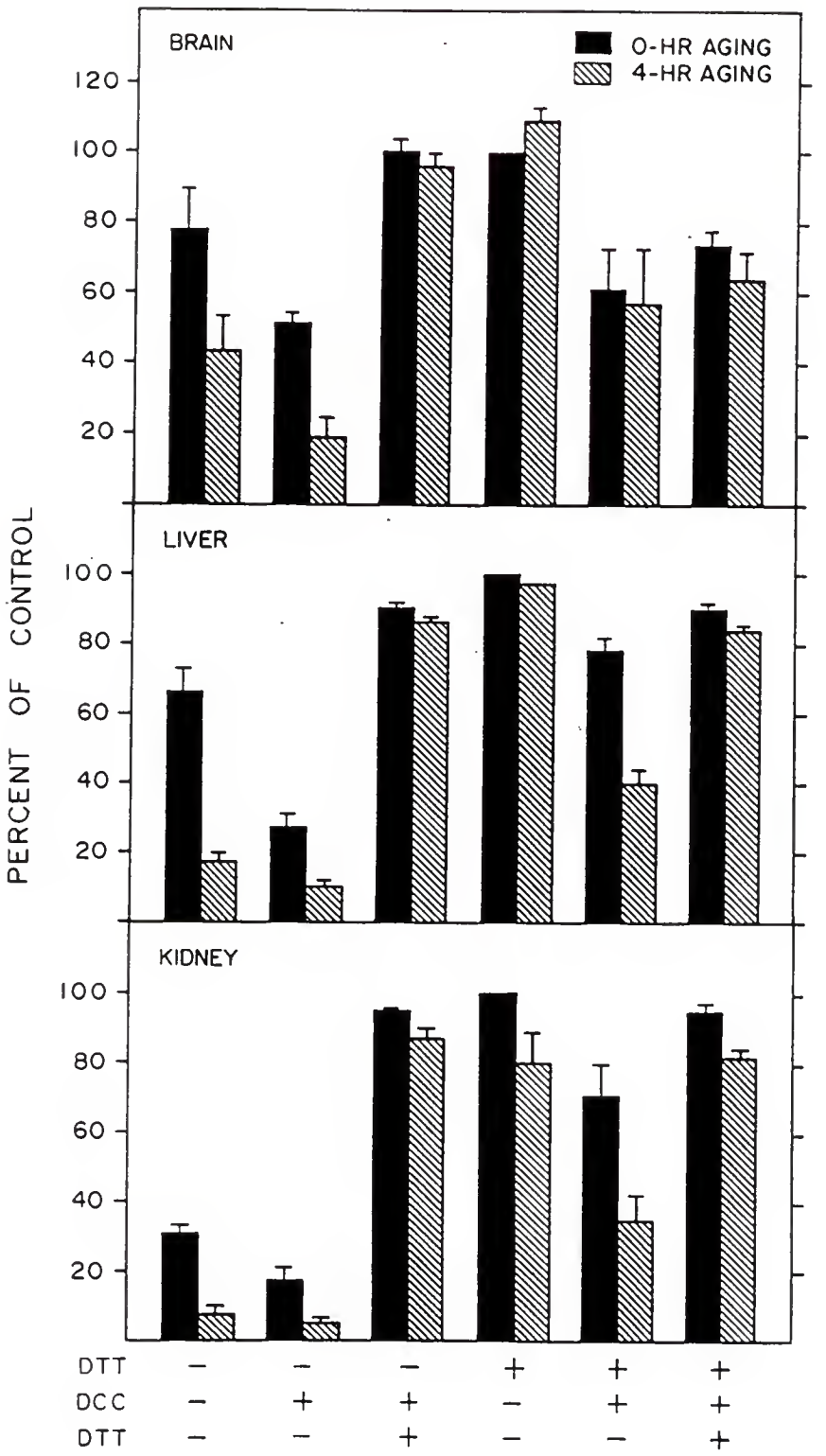
In an attempt to further characterize the endogenous factor(s) involved in the reversible in vitro down-regulation of unoccupied glucocorticoid receptors, cytosol preparations were subjected to a dextran-coated charcoal (DCC) pretreatment with or without DTT addition (before and/or after DCC). The effect of temperature on this DTT-reversible binding loss was also examined under each of the possible conditions by aging half of the cytosol from each group for an additional 4 hr at 22 C prior to incubation with labeled steroid.

Figure 3-2. Temperature-dependent reversible loss of glucocorticoid binding capacity of unoccupied Type II glucocorticoid receptors in the presence of molybdate and absence of DTT after extended periods of incubation at 22 C. Open bars represent brain cytosol prepared in HEPES buffer containing 20 mM molybdate and incubated at 22 C in the absence of steroid prior to a 24 hr incubation with 20 nM [3H]DEX +/- 4 uM [1H]DEX at 0 C. Hatched bars represent the addition of DTT (2 mM final concentration) to the preincubated cytosol just prior to the initiation of steroid incubations. Solid bars represent the addition of 2 mM DTT prior to the incubation at 22 C in the absence of steroid followed by the steroid incubations. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the 0 hr control group (310 fmole/mg cytosol protein) and represents the mean +/- S.E.M. of 3 independent replications.


Percent of Control
100
75
50
25
0
2
5
9
21
30
Duration of 22°C Aging (hours)

Finally, liver and kidney cytosol preparations were treated in an identical manner for comparative purposes since these tissues reportedly have a higher endogenous reducing potential (Granberg and Ballard, 1977). For brain, the DCC pretreatment in the absence of DTT additions or subsequent 22 C aging led to a significant decrease (approximately 40%) in the binding capacity for [3H]DEX (Figure 3-3). There was also an increase in the rate of loss of binding capacity at 22 C. When DTT was added back to cytosol after DCC pretreatment there was virtually a 100% recovery of binding capacity seen in cytosol treated with DTT but not DCC. When DTT was added to cytosol prior to DCC treatment, there was still a loss of binding capacity to a level similar to that seen if no DTT was present before DCC. However, there was no significant additional loss of binding capacity after a subsequent 22 C aging as was the case for the DTT-, then DCC-treated group. Interęstingly, when DTT was added both before and after the DCC pretreatment, there was only a partial restoration of the total binding capacity although there was again very little loss of binding capacity associated with aging. Results for liver and kidney were clearly different from those obtained for brain and surprizingly different from what was expected based on the findings of other workers regarding the endogenous reducing potential for these tissues. The degree of loss of binding capacity seen in groups not pretreated with DCC, DTT or 22 C aging was greater in liver and much greater in kidney than that observed for brain. In addition, the rate of loss of binding capacity for these same groups during 22 C aging was greatly enhanced for both tissues when compared to brain. However, similar to brain cytosol, DCC pretreatment led to a significant decrease in binding capacity that was fully reversible upon addition of

Figure 3-3. Effects of dextran-coated charcoal pretreatment and DTT on glucocorticoid binding capacity and thermal stability of unoccupied Type II glucocorticoid receptors from brain, liver and kidney. Concentrated cytosol was prepared in HEPES buffer plus 20 mM molybdate and then diluted with either additional molybdate-containing buffer or molybdate-containing buffer plus 20 mM DTT (2 mM final concentration) prior to a 20 min incubation in the presence or absence of dextran- coated charcoal at 0 C. Cytosols were then further diluted with either additional molybdate-containing buffer or molybdate-containing buffer plus 20 mM DTT (2 mM final concentration) and then incubated at 22 C for either 0 (solid bars) or 4 (hatched bars) hr in the absence of steroid prior to incubation with 20 nM [3H]DEX +/- 4 uM [1H]DEX at 0 C for 24 hr. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the 0 hr control groups (300, 281, and 341 fmole/mg protein for brain, liver and kidney cytosols, respectively) and represents the mean + S.E.M. of 5 independent replications and 3 independent replications for liver and kidney.


BRAIN
0-HR AGING
4-HR AGING
LIVER
KIDNEY
PERCENT OF CONTROL
120
100
80
60
40
20
DTT – – – + + +
DCC – + + – + +
DTT – – + – – +

DTT. Unlike brain cytosol, however, both liver and kidney cytosols exhibited a significant degree of protection from DCC-induced binding loss when pretreated with DTT. However, DTT pretreatment preceding DCC pretreatment of liver and kidney cytosol appeared to be less effective in preventing further binding loss during 22 C aging than was found to be the case for brain cytosol treated with DTT before and after DCC pretreatment was significantly higher for liver and kidney than it was for brain.

Since the chemical nature of the sulfhydryl up- and down-regulation process was unknown, an investigation was made to determine if the process involved any major, easily measurable changes in the molecular structure of the receptor (i.e. receptor subunit association or dissociation or major conformational changes). The aim of this experiment was to compare the sedimentation properties of up- and down-regulated unoccupied glucocorticoid receptors from brain using sucrose density gradient analysis. Cytosol was up-regulated by incubating with DTT at 0 C prior to the gradient run on sucrose gradients prepared and DTT. Cytosol was down-regulated by incubating without DTT at 22 C for 4 hr prior to the gradient run on sucrose gradients prepared without DTT. All cytosol groups and sucrose gradients contained molybdate. After an 18 hr gradient run, gradients of both treatment groups were subjected to fractionation followed by postlabeling of the individual fractions with [3H]DEX. Steroid incubation conditions were identical for both groups except that DTT had to be added back to each fraction of the down-regulated group to allow for up-regulation so that binding could be determined. The profiles representing the specific binding determined for each fraction indicated

that both forms sediment in one major peak of approximately 9.2 S (Figure 3-4). The results therefore provide little evidence for any major conformational changes in the receptor associated with sulfhydryl regulation of binding capacity under these conditions.

To determine if the changes in glucocorticoid binding capacity associated with sulfhydryl down-regulation involve merely a decrease in the maximal binding or, instead, a modification of the receptor binding affinity, Scatchard analysis of DEX binding was performed for cytosol either containing or not containing DTT (Figure 3-5). The addition of DTT led to an apparent increase in both the affinity and maximal binding. Equilibrium binding constants were also determined after 4 hr of 22 C aging of the cytosol with and without DTT prior to steroid incubation. This aging only had a significant effect on the parameters determined for non-DTT-containing cytosol, leading to a further decrease in both maximal binding and apparent affinity.

If the apparent reduction in affinity associated with sulfhydryl down-regulation as determined by Scatchard analysis is real, it is likely to be due to either a decrease in the rate of steroid association, an increase in the rate of steroid dissociation or both. Each of these kinetic parameters were investigated. To study the effect of reducing reagents on the rate of steroid dissociation from the glucocorticoid receptor, cytosol was first prelabeled with [3H]DEX in the presence of molybdate and DTT prior to being run on a Sephadex G-25 column equilibrated in non-DTT-containing buffer to remove both the free steroid and the DTT. DTT was then added back to half of the preparation while the other half was appropriately diluted with non-DTT-containing buffer. As illustrated in Figure 3-6, there was no significant

Figure 3-4. Sucrose density gradient analysis of sulfhydryl-reduced and sulfhydryl-oxidized unoccupied Type II glucocorticoid receptors. Brain cytosol prepared in the absence of DTT (HEPES buffer plus 20 mM molybdate) was either incubated in the absence of steroid for 4 hr at 22 C (solid squares) or supplemented with DTT (solid triangles, 2 mM final concentration) and stored at 0 C for 4 hr. Aliquots (400 ul) from both cytosol samples were then sedimented through 5-20% linear sucrose gradients in a SW 50.1 rotor at 200,000 x g for 18 hr. Samples containing DTT (solid triangles) were run on gradients prepared with HEPES buffer plus 20 mM molybdate and 2 mM DTT, whereas samples not containing DTT (solid squares) were run on gradients prepared with HEPES buffer containing only 20 mM molybdate. Fractions were collected directly into ice-cold tubes containing either [3H]DEX in HEPES buffer plus 20 mM molybdate and 2 mM DTT (solid triangles) or [3H]DEX plus HEPES buffer plus 20 mM molybdate and 20 mM DTT (solid squares, 2 mM DTT final concentration). The individual fractions were then incubated for 24 hr at 0 C prior to bound-free steroid separations on LH-20 columns. Results presented here represent the mean of 2 independent replications.

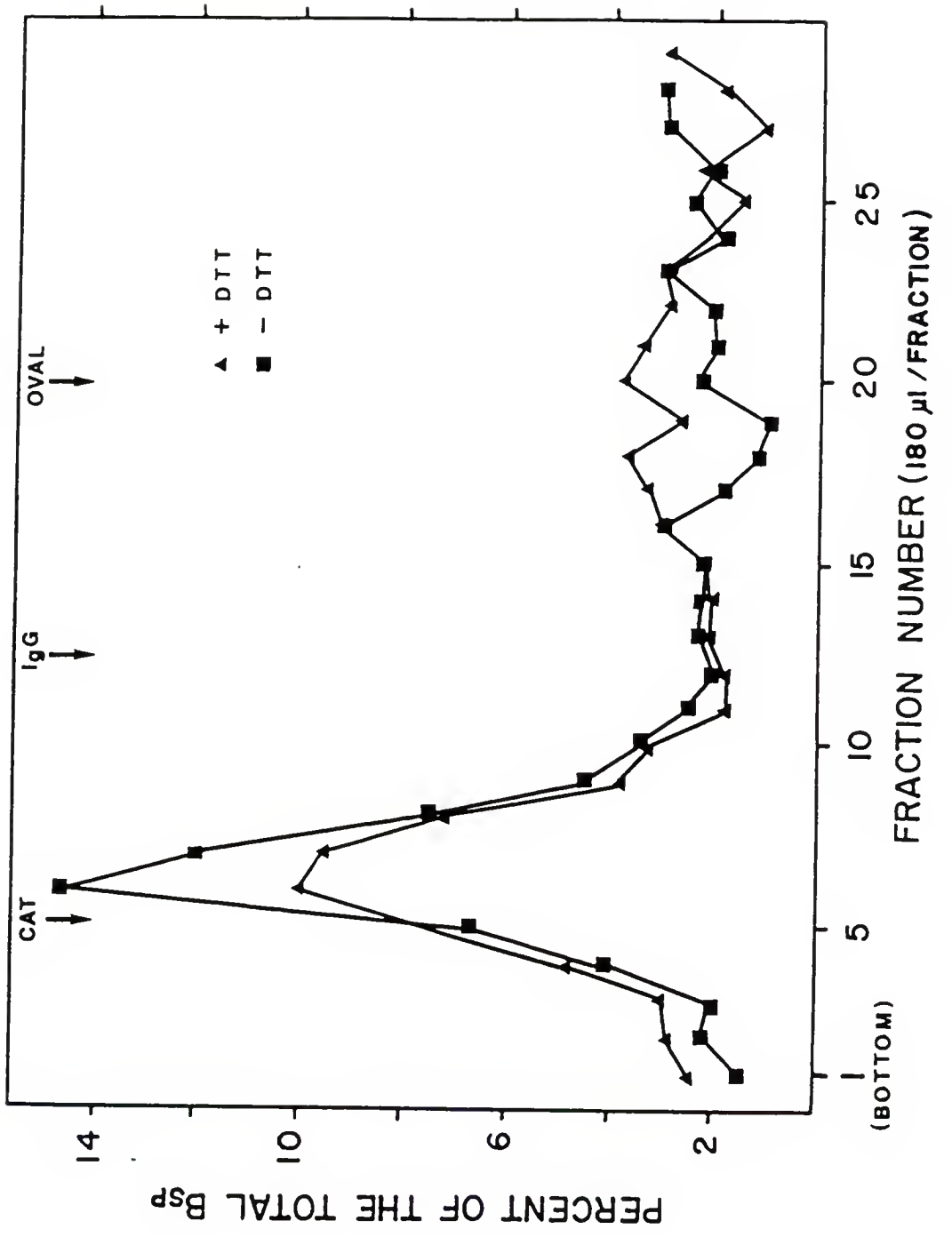

PERCENT OF THE TOTAL B_{SP}
14
10
6
2
CAT
IgG
OVAL
▲ + DTT
■ – DTT
1 (BOTTOM)
5
10
15
20
25
FRACTION NUMBER (180 µl / FRACTION)

Figure 3-5. Scatchard analysis of [3H]DEX binding to Type II glucocorticoid receptors. Brain cytosol was prepared in HEPES buffer containing 20 mM molybdate plus (circles) or minus (triangles) 2 mM DTT and with (solid symbols) or without (open symbols) a 4 hr incubation at 22 C in the absence of steroid prior to incubation with [3H]DEX +/- 200-fold [1H]DEX at 0 C for 24 hr. Bound-free steroid separations were performed on Sephadex G-25 columns.

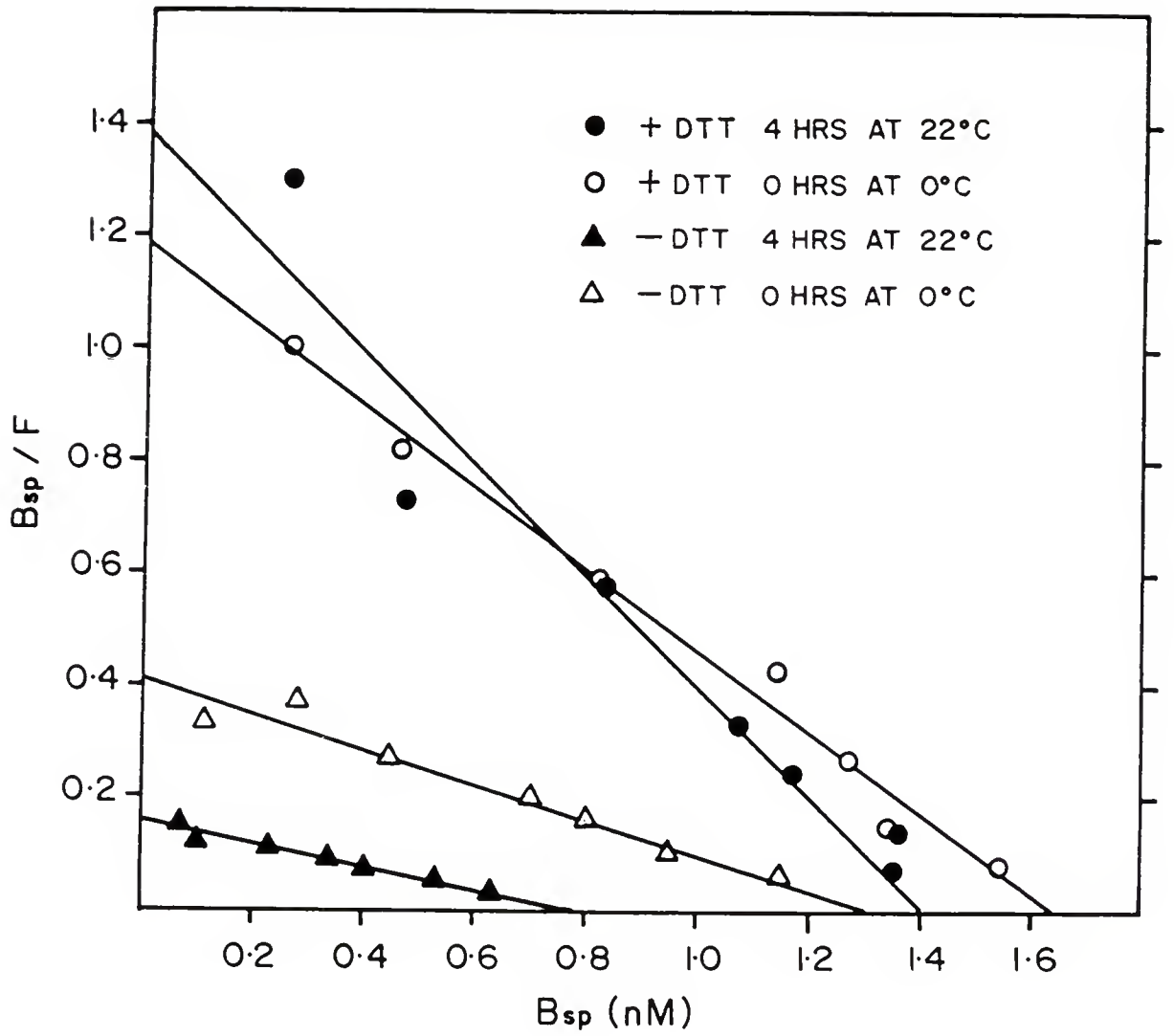
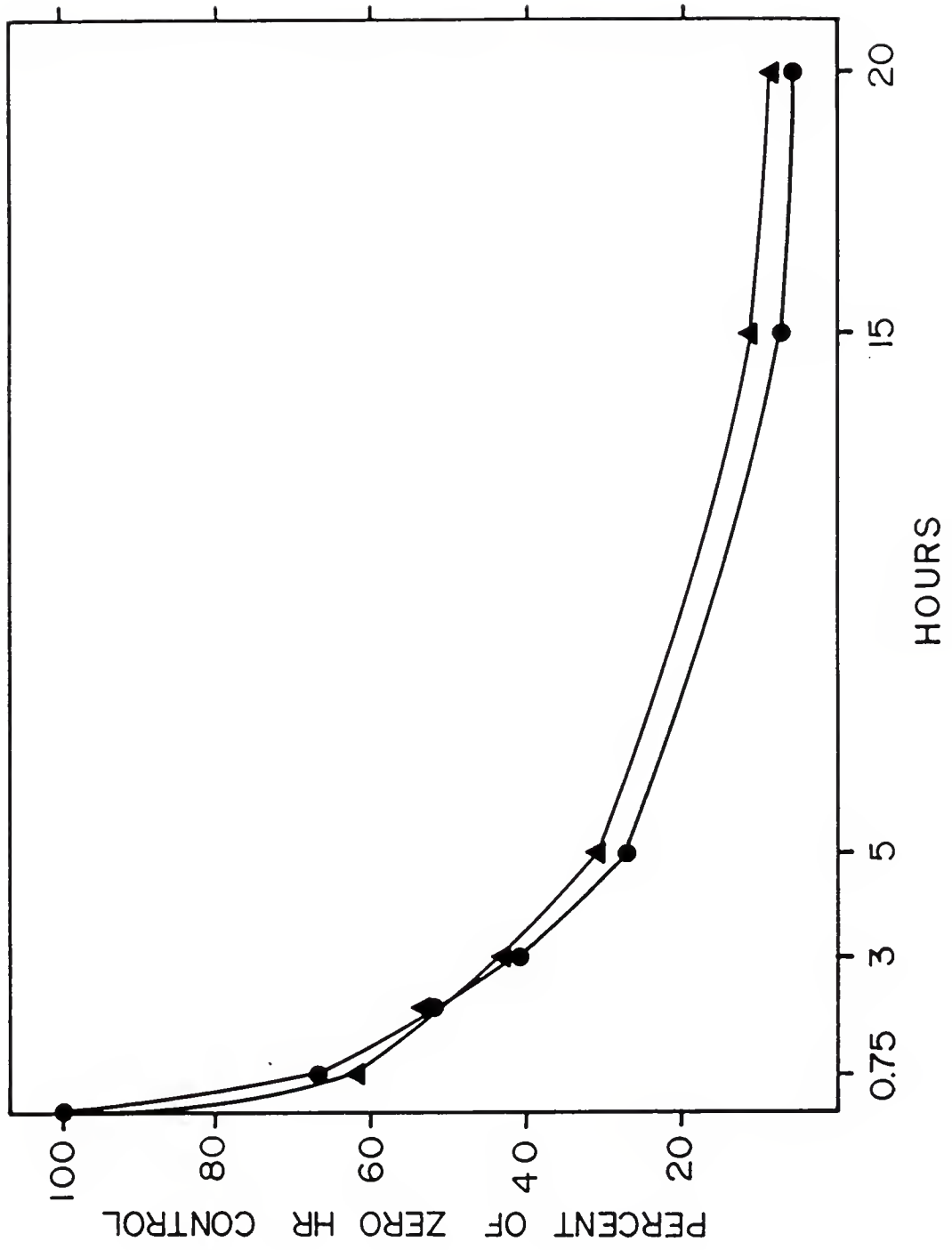

+ DTT 4 HRS AT 22°C
+ DTT 0 HRS AT 0°C
— DTT 4 HRS AT 22°C
— DTT 0 HRS AT 0°C
Bsp / F
Bsp (nM)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6

Figure 3-6. Effect of DTT on the rate of dissociation at 22 C of [3H]DEX from Type II glucocorticoid receptors. Brain cytosol prepared in HEPES buffer plus 20 mM molybdate and 2 mM DTT was incubated with 40 nM [3H]DEX for 50 hr at 0 C. Labeled cytosol was then run on a jacketed Sephadex G-25 column (1 x 50 cm) at -4 C to remove both the free steroid and the DTT. Cytosol was then incubated in the presence of 10 uM [1H]DEX at 22 C either with (circles) or without (squares) 2 mM DTT. Aliquots were removed periodically for bound-free steroid separations on Sephadex LH-20 columns.


PERCENT OF ZERO HR CONTROL
100
80
60
40
20
0.75
3
5
15
20
HOURS

differences in the rate of dissociation between receptors in the presence or absence of DTT. Although an attempt was made to study the effect of DTT on the rate of association of [3H]DEX to glucocorticoid receptors, the results of this experiment were confounded by the fact that the binding capacity of cytosol lacking DTT was undergoing a time-dependent loss that did not occur in the presence of DTT.

A series of experiments were next performed to further characterize the sulfhydryl up and down-regulation of glucocorticoid binding capacity by studying the reactivity of the essential sulfhydryl group(s) with a variety of known reversible and irreversible sulfhydryl-reactive agents. The first experiment examined the effects of a 1 hr incubation of unlabeled cytosol with 1 mM dithionitrobenzoic acid (DTNB), n-ethylmaleimide (NEM), iodoacetamide, cystamine, glutathione disulfide (GSSG) and para-chloromercurisulfonate (PCMS) with or without the subsequent addition of 10 mM DTT on glucocorticoid binding capacity as measured by [3H]DEX binding (Figure 3-7). DTNB and cystamine led to a virtual total loss in binding capacity which was completely reversible upon DTT addition. GSSG resulted in only a partial loss in binding capacity, but this loss was also completely reversible upon DTT addition. NEM, iodoacetamide and PCMS led to varying degrees of loss which were not completely reversible with DTT. In a follow-up experiment, conditions identical to those described in the previous experiment were used to investigate the effects on glucocorticoid binding capacity of several additional sulfhydryl reactive reagents including iodoacetic acid, sodium-m-periodate, sodium-p-periodate, 6,6'-dithionicotinic acid (DTNT), methyl methane thiosulfonate (MMTS) and mercuric chloride (Figure 3-8). Iodoacetic acid was relatively

Figure 3-7. Effects of 0 C incubation of cytosol with various known reversible and irreversible sulfhydryl-reactive reagents on Type II glucocorticoid receptor binding capacity with and without subsequent addition of excess DTT. Brain cytosol prepared in HEPES buffer plus 20 mM molybdate was incubated at 0 C for 1 hr with either additional HEPES buffer dilution (BUFF) or a 1 mM final concentration of dithionitro- benzoic acid (DTNB), n-ethyl maleimide (NEM), iodoacetamide (IAM), cystamine (CYS), glutathione dissulfide (GSSG) and para-chloro- mercurisulfonate (PCMS). Cytosol samples were then supplemented with (solid bars) or without (open bars) and excess of DTT (10 mM final concentration) prior to incubation with 20 nM [3H]DEX +/- 4 um [1H]DEX at 0 C for 40 hr. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the BUFF control group after addition of DTT (317 fmol/mg protein) and represents the mean +/- S.E.M. of 3 independent replications.

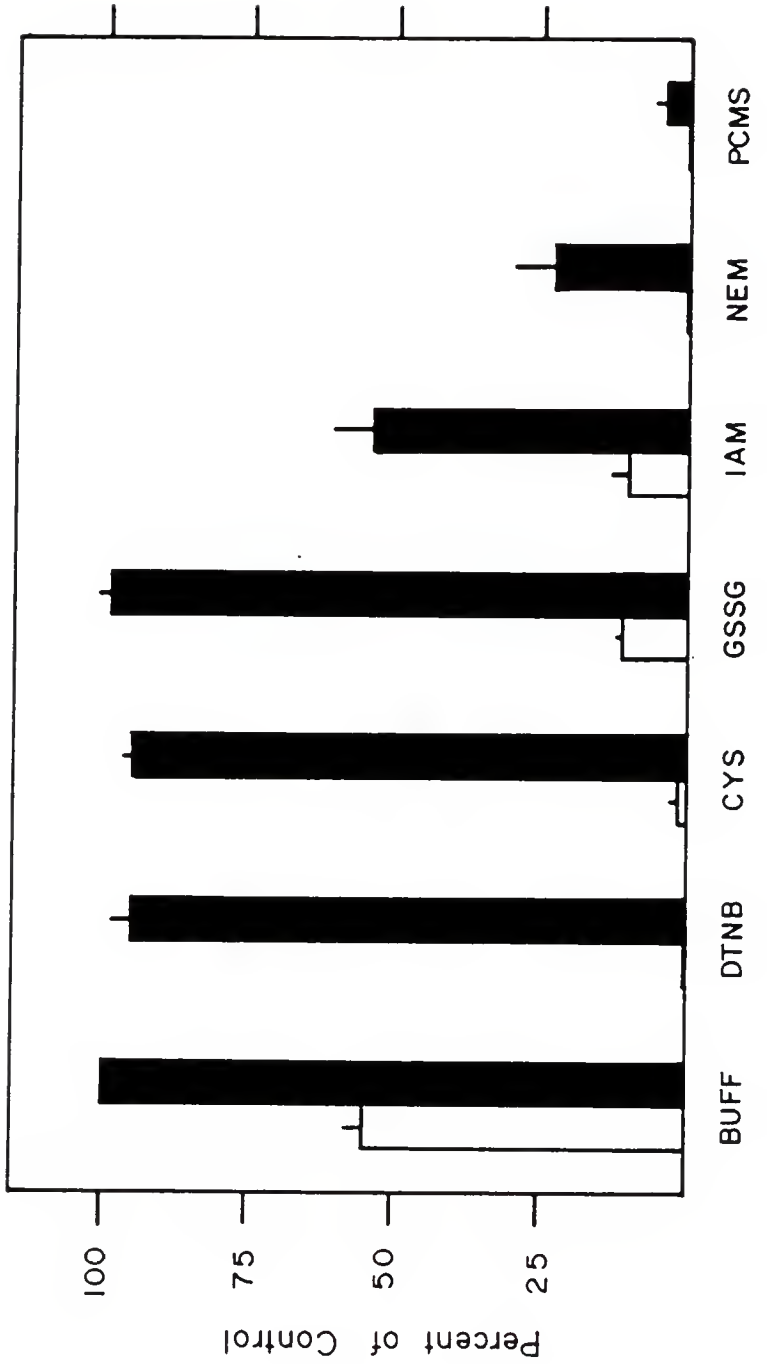
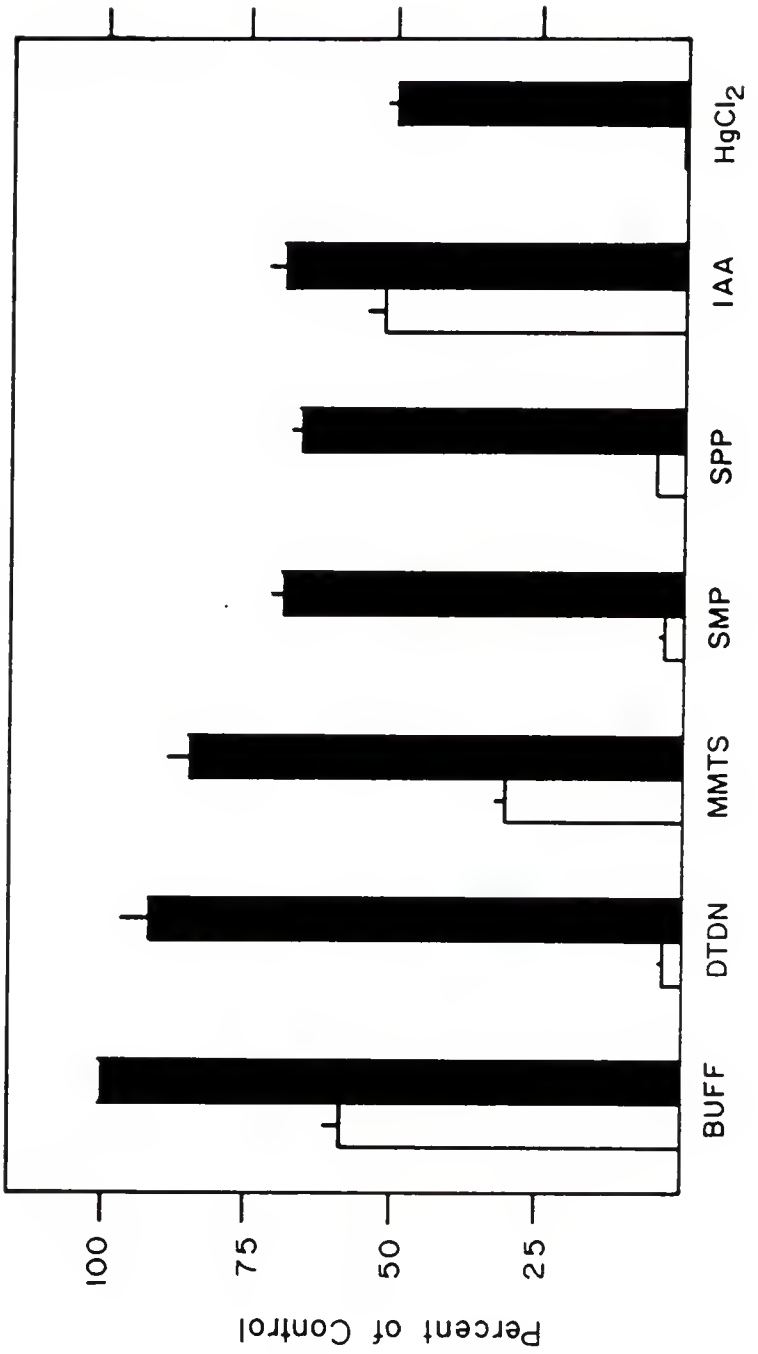

Percent of Control
100
75
50
25
BUFF
DTNB
CYS
GSSG
IAM
NEM
PCMS

Figure 3-8. Effects of 0 C incubation of cytosol with additional known reversible and irreversible sulfhydryl-reactive reagents on Type II glucocorticoid receptor binding capacity with and without subsequent addition of excess DTT. Brain cytosol prepared in HEPES buffer plus 20 mM molybdate was incubated at 0 C for 1 hr with either additional HEPES buffer (BUFF) or a 1 mM final concentration of 6,6'-dithionicotinic acid (DTDN), methyl methane thiosulfonate (MMTS), sodium-m-periodate (SMP), sodium-p-periodate (SPP), iodoacetic acid (IAA) or mercuric chloride (HgCl2). Cytosol samples were then supplemented with (solid bars) or without (open bars) an excess of DTT (10 mM final concentration) prior to incubation with 20 nM [3H]DEX +/- 4 um [1H]DEX at 0 C for 40 hr. Bound-free steroid separations were performed on Sephadex G-25 minicolumns. Specfic binding is expressed as percent of the BUFF control group after addition of DTT (347 fmol/mg protein) and represents the mean +/- S.E.M. of 3 independent replications.


Percent of Control
100
75
50
25
BUFF
DTDN
MMTS
SMP
SPP
IAA
$HgCl_2$

ineffective in reducing the binding capacity, possibly due to the pH of the system or the low concentration used, but it did produce some irreversible losses. There was no difference between the two isomers of sodium periodate which were intermediate in reducing binding capacity which was not completely DTT-reversible. DTNT resulted in nearly a total loss of binding capacity which was completely reversible while MMTS was much less effective in reducing binding capacity. However, total reversibility with DTT in the case of MMTS was less certain than for DTNT. Finally, exposure to mercuric chloride resulted in a complete loss of binding capacity which was partially reversible.

The next step was to investigate the ability of DTT-reversible sulfhydryl reactive reagents to protect unoccupied receptors from irreversible inactivation due to non-DTT-reversible sulfhydryl reactive reagents. This experiment was designed to determine if unoccupied receptors, down-regulated by endogenous sulfhydryl oxidizing factors, were more or less protected from irreversible sulfhydryl reactive reagents than were receptors down-regulated by the DTT-reversible reagents used in the previous experiments. This was an effort to possibly shed light on the nature of the down-regulated form in vivo as well as to provide information on a possible means of "trapping" up- and/or down-regulated forms of the receptor so that the true status of up- and down-regulated receptors in vivo may someday be determined. This experiment involved first, a 1 hr incubation of unlabeled cytosol at 0 C with DTNB, cystamine, GSSG or no reagent for the DTT-reversible down-regulation of the unoccupied receptors. This was followed by an additional 1 hr incubation of each of the above groups with either NEM, iodoacetamide, mercuric chloride or no reagent. This second incubation

with DTT-irreversible sulfhydryl reactive reagents was followed by further dilution with or without DTT prior to [3H]DEX binding. Binding data obtained for all 32 possible groups are illustrated in Figure 3-9. DTNB and cystamine provided almost complete protection against irreversible losses caused by either NEM or iodoacetamide. GSSG, on the other hand, provided only a slight protection against the NEM- and iodoacetamide-induced losses in binding capacity. Interestingly, none of the reversible reagents provided any significant degree of protection against the irreversible effects of mercuric chloride when compared to the control that subsequently received mercuric chloride. These later findings imply that either mercuric chloride is ineffective against the endogenous down-regulated form, while being effective against the forms down-regulated via reaction with DTNB, cystamine or GSSG, or that mercuric chloride's effects are relatively nonspecific against both up- and down-regulated receptors.

If one makes the assumption that mercuric chloride is acting specifically on the sulfhydryl up-regulated form of the receptor, a possibility implied by the results of the previous experiment, then one would expect the incubation of cytosol containing quantitatively known populations of both up- and down-regulated glucocorticoid receptors with mercuric chloride, followed by addition of excess DTT (to neutralize the reaction) at various time intervals, to result in a decreasing binding capacity that approaches asymptotically the level of down-regulated receptors. The results, shown in Figure 3-10, basically confirm this. In the presence of 2 mM mercuric chloride, the binding capacity was reduced by around 35% of maximum, a level approximating that of the down-regulated receptors determined under similar conditions.

Figure 3-9 . The ability of DTT-reversible sulfhydryl reagents to protect unoccupied Type II glucocorticoid receptors from irreversible inactivation due to non DTT-reversible sulfhydryl-reactive reagents. Brain cytosol prepared with HEPES buffer plus 25 mM molybdate was incubated at 0 C with either additional HEPES buffer (BUFF) or a 1 mM final concentration of dithionitrobenzoic acid (DTNB), cystamine (CYS) or glutathione dissulfide (GSSG). Cytosol samples were then incubated at 0 C for a second hr with either additional HEPES buffer (BUFF) or a 2 mM final concentration of n-ethyl maleimide (NEM), iodoacetamide (IAM) or mercuric chloride (HgCl2). Each subset of cytosol was then incubated at 0 C for an additional 1 hr prior to the addition of either HEPES buffer (open bars) or HEPES buffer containing excess DTT (20 mM final concentration, solid bars) followed by incubation at 0 C for 40 hr with 20 nM [3H]DEX +/- 4 um [1H]DEX. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the BUFF-BUFF plus DTT control group (302 fmol/mg protein) and represents the mean +/- S.E.M. of 3 independent replications.

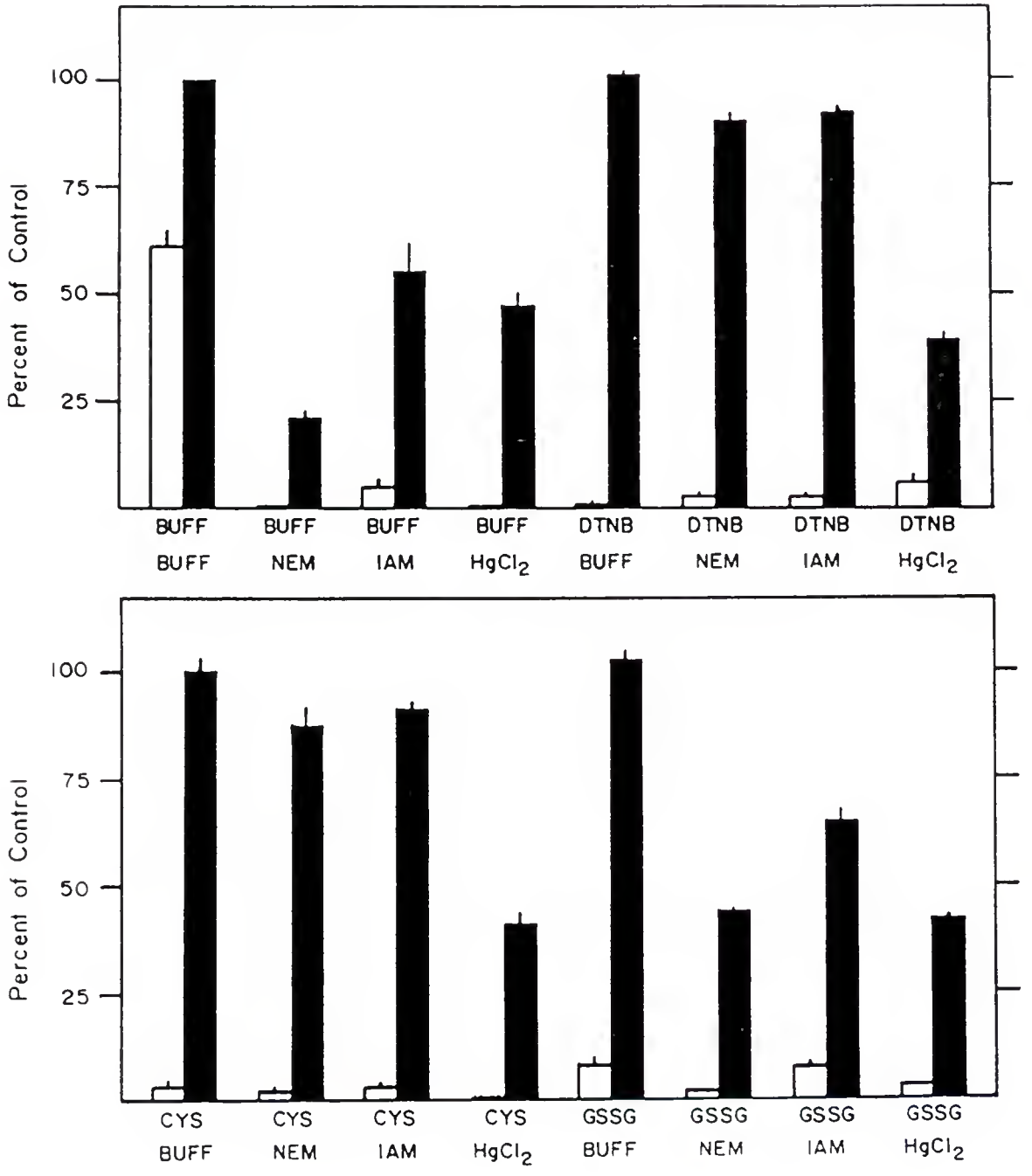
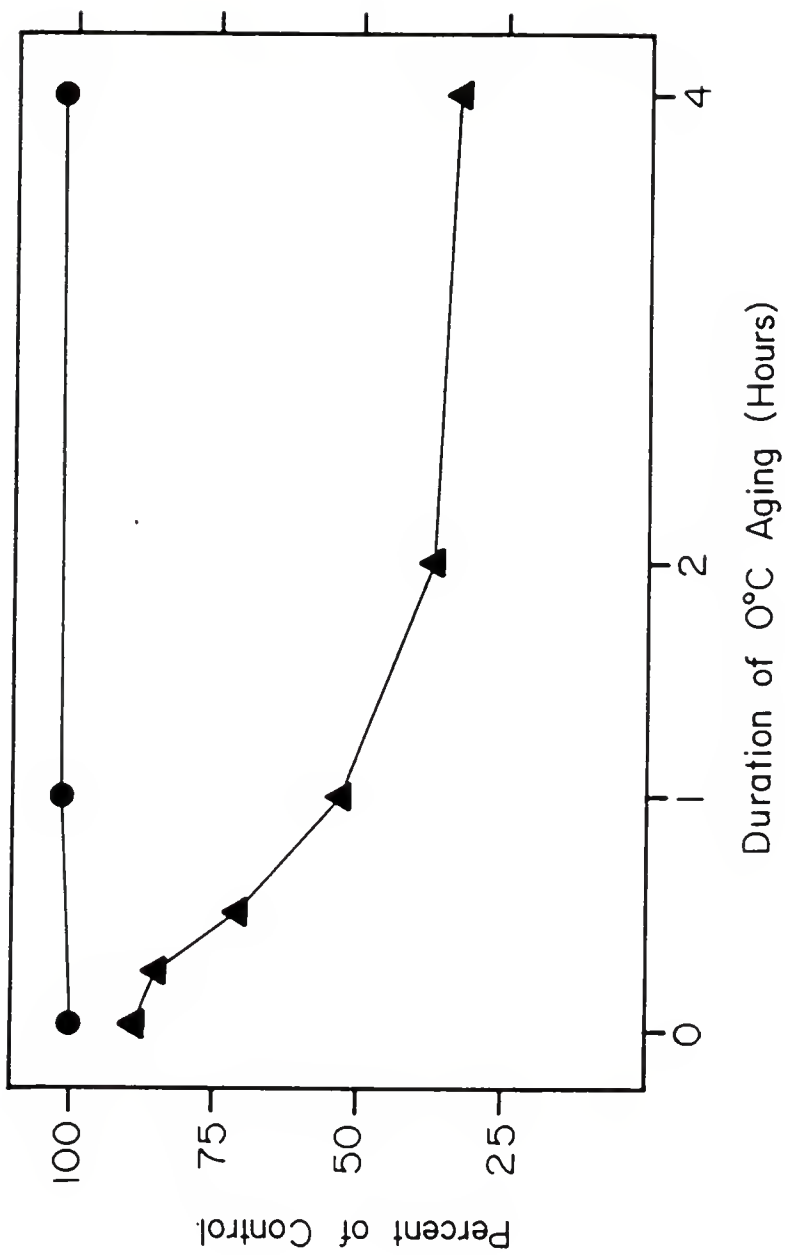

Percent of Control
100
75
50
25
BUFF BUFF
BUFF NEM
BUFF IAM
BUFF $HgCl_2$
DTNB BUFF
DTNB NEM
DTNB IAM
DTNB $HgCl_2$
Percent of Control
100
75
50
25
CYS BUFF
CYS NEM
CYS IAM
CYS $HgCl_2$
GSSG BUFF
GSSG NEM
GSSG IAM
GSSG $HgCl_2$

Figure 3-10. Time course of the effects of 1 mM mercuric chloride on Type II glucocorticoid receptor binding capacity in the absence of steroid and with the subsequent addition of DTT. Brain cytosol prepared with HEPES buffer plus 20 mM molybdate was either brought to a final concentration of 1 mM mercuric chloride (triangles) or was diluted with an appropriate volume of HEPES buffer (circles). Cytosols were then incubated at 0 C for the times indicated prior to addition of excess DTT (10 mM final concentration) followed by incubation at 0 C for 40 hr with 20 nM [3H]DEX +/- 4 um [1H]DEX. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as the percent of the 0 hr control (no mercuric chloride) (297 fmol/mg protein) and represents the mean +/- S.E.M. of 3 independent replications.

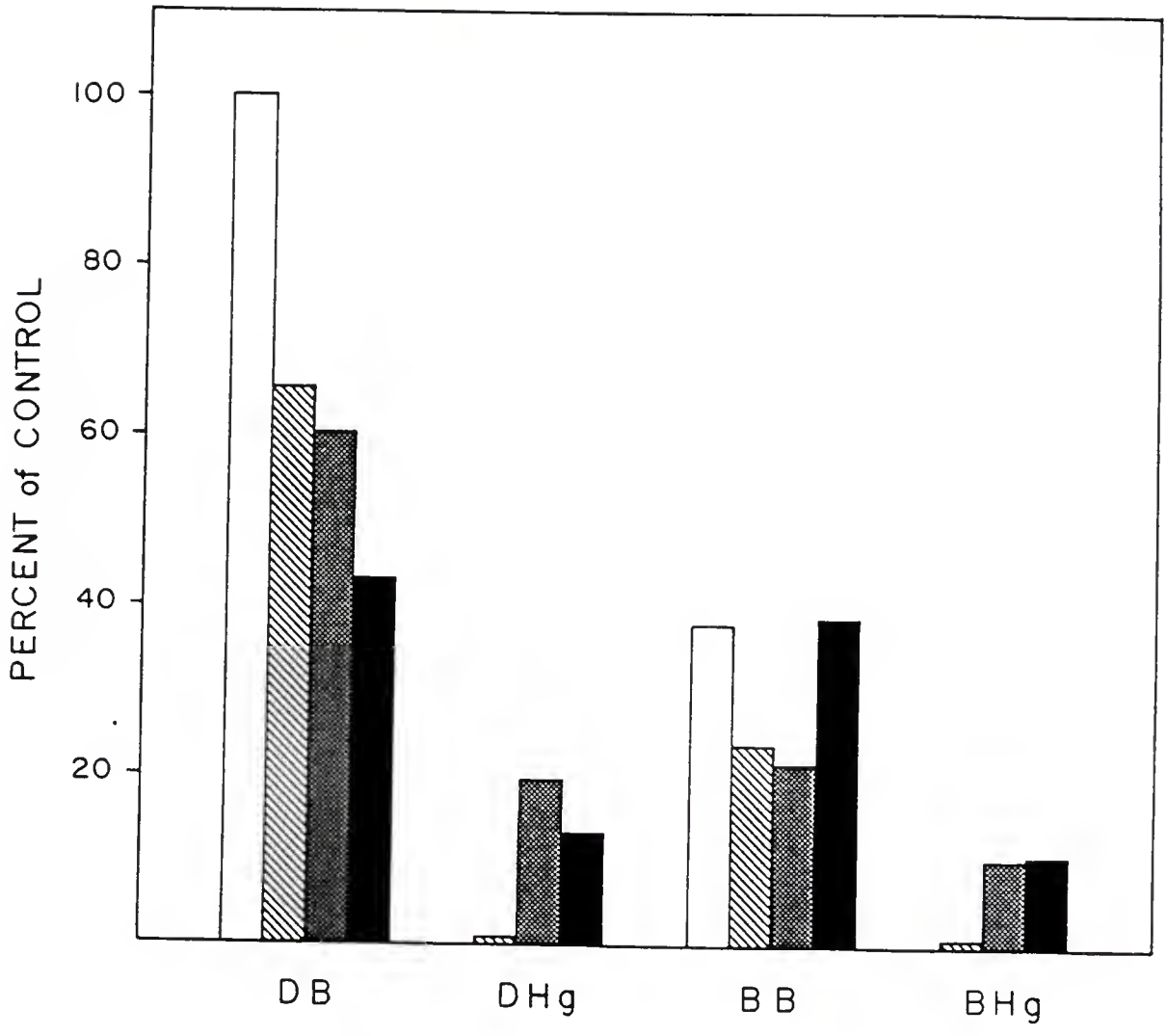

100
75
50
25
Percent of Control
0
1
2
4
Duration of 0°C Aging (Hours)

The next experiment was designed to determine if DTT-reversible sulfhydryl reactive reagents were capable of interacting with prelabeled glucocorticoid receptors and affecting the rate of steroid dissociation, inactivation, etc. for the steroid receptor complex. Cytosol was prepared in the absence of any sulfhydryl reactive or reducing reagents and prelabeled with 20 nM [3H]DEX for 40 hr at 0 C. After a bound/free separation on Sephadex G-25 columns, cytosol was incubated with 1 mM concentration of DTNB, cystamine, GSSG or an appropriate dilution of HEPES buffer devoid of any sulfhydryl reagents for 4 hr at 0 C in the presence or absence of 4 uM [1H]DEX. The results from this experiment (data not shown) confirmed expectations that these DTT-reversible reagents could only affect the steroid binding properties of unoccupied glucocorticoid receptors since no enhancement of the rate of dissociation of [3H]DEX was evident. It was evident, however, that interactions between these reagents and receptors unoccupied by virtue of normal steroid dissociation did reversibly prevent steroid rebinding.

When the previous experiment was replicated using mercuric chloride, instead of known DTT-reversible reagents, very different results were obtained. When cytosol containing both [3H]DEX-labeled (up-regulated) receptors and down-regulated unoccupied receptors was incubated in the presence of 1 mM mercuric chloride for 4 hr, there was a near total loss of receptor binding. Upon addition of DTT, and after subsequent reincubation with [3H]DEX, binding equivalent to the population of down-regulated unoccupied receptors reappeared (data not shown), indicating the possibility that bound steroid was not protective of the inactivating effects of the mercury, but that the down-regulated form was somehow protected from such inactivation.

The fact that the in vivo down-regulated receptor is protected from inactivation by mercurial reagents, while steroid binding or reversible interactions with DTNB, cystamine and GSSG are ineffective in protecting the receptor from inactivation could have potentially important functional implications for the role and/or the mechanism of down-regulation in vivo if proven to be true. The aim of the next experiment was to further investigate this phenomenon by looking at the effects of mercuric chloride on receptors that had been completely up-regulated and labeled in the presence of DTT as well as a mixed population as was studied before. If the original theory is correct, one would expect to see a total irreversible loss of the DTT-incubated samples while incubation of the mixed population would result in a total binding loss that was partially restorable (representing the down-regulated unoccupied receptors) upon addition of excess DTT. The experiment was also different from the previous experiment in that both plus and minus DTT cytosol samples were run through Sephadex G-25 columns equilibrated with buffer devoid of DTT prior to incubation with mercuric chloride. In addition to removing DTT, this column run also removed endogenous small thiols that compete or otherwise interfere with the mercury-thiol reaction. After these Sephadex G-25 column runs, cytosol was incubated with mercuric chloride, followed by another G-25 column run and subsequent reincubation with [3H]DEX in the presence of high concentrations of DTT. The results provided evidence that the original theory regarding the selective action of mercury against up-regulated receptors only was flawed (Figure 3-11). To begin with, the incubation with mercuric chloride did not lead to a complete loss of previously bound receptors as previously thought. Although this

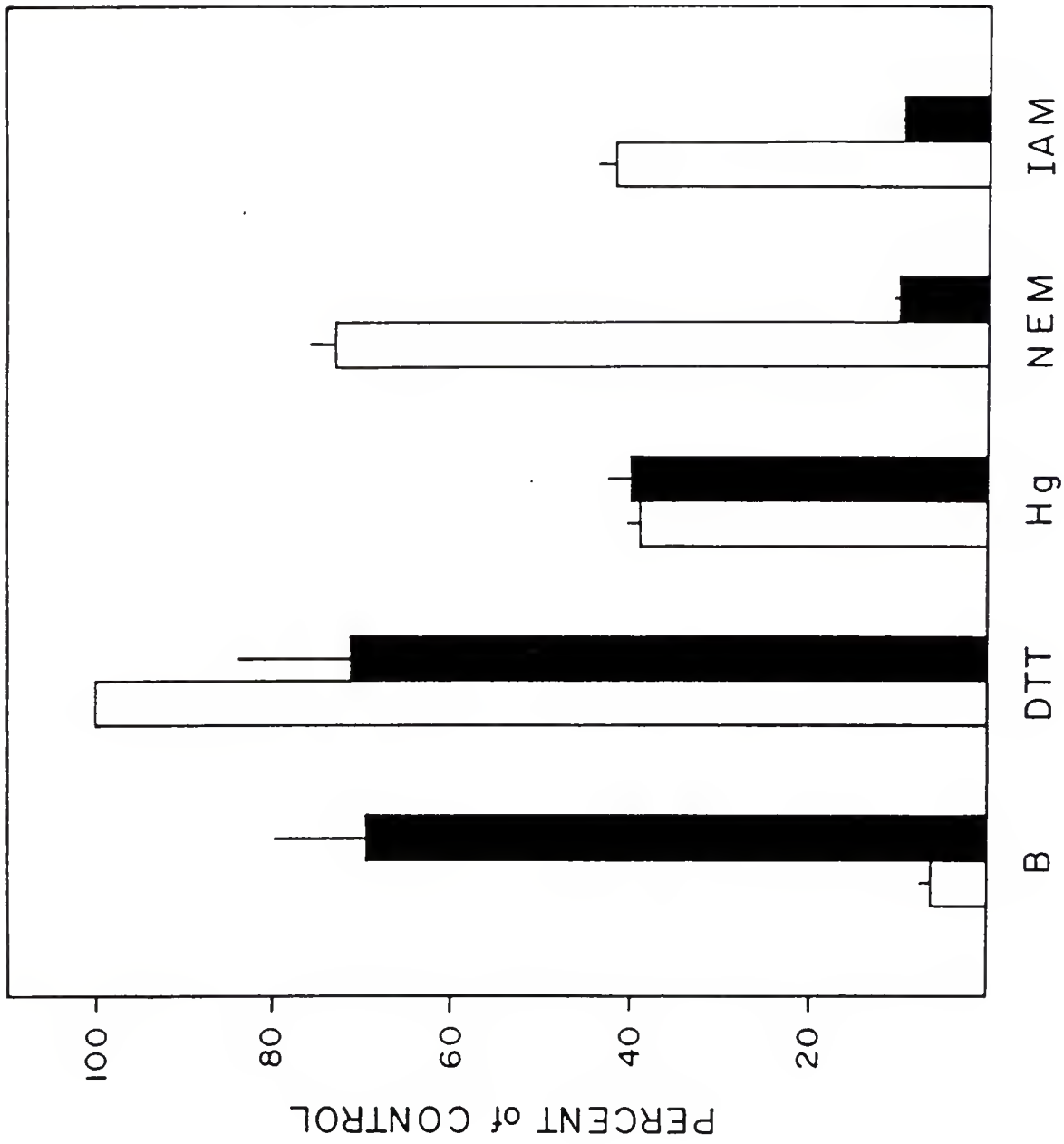
Figure 3-11. Effects of mercuric chloride on occupied type II glucocorticoid receptors labeled in the presence or absence of DTT. Brain cytosol prepared in HEPES buffer plus 25 mM molybdate was divided into half and supplemented with either HEPES buffer (B) or HEPES buffer plus DTT (D, 10 mM final concentration). Both groups were then incubated at 0 C for 4 hours to allow for reduction of all reversibly oxidized sulfhydryls in the DTT group. Both groups were then run through Sephadex G-25 columns to remove DTT and any small endogenous thiols present. Each group was then treated with either HEPES buffer plus mercuric chloride (DHg, BHg; 2 mM final concentration) or HEPES alone (DB, BB) and then incubated for 4 hours at 0 C. Aliquots of the different cytosolic groups were run directly on Sephadex G-25 columns for bound-free separations (hatched bars). HEPES buffer plus DTT (10 mM final concentration) and 20 nM [3H]TA + 4 uM [1H]TA was added to the remainder of each of the cytosol groups and another aliquot was immeadiately taken from each for bound-free separation (screened bars). The remaining cytosol was incubated for an additional 40 hr at 0 C prior to running the final bound-free separation (solid bars). The binding is expressed as percent of the initial control binding of the DTT only group (DB open bar, 340 fmole/mg protein) and represents the mean of 2 replications. All binding is corrected for sequencial dilutions.


PERCENT of CONTROL
100
80
60
40
20
DB
DHg
BB
BHg

appeared to be the case when the first bound/free separations were performed after the incubation with mercuric chloride, but prior to the readdition of DTT, it was discovered that addition of excess DTT caused an immeadiate "restoration" of part of the binding, too fast to be accounted for by rebinding of dissociated receptors or up-regulation and subsequent binding of down-regulated forms. It was obvious from changes in the physical appearance of the cytosol after the addition of mercuric chloride and again after the subsequent addition of excess DTT that the mercury was causing an aggregation of cytosolic protein leading to a faintly cloudy suspension. Dissolution of the suspension occurred instantly upon addition of DTT indicating that a portion of the "lost" binding was simply complexed or trapped in the suspension, unable to pass through Sephadex G-25 gel. Perhaps even more notable was that there was no significant increase in binding in either the DTT- or non-DTT-pretreated cytosols from the time that DTT was readded after mercury treatment until 40 hours later. In other words, the mercury seemed to affect the receptor binding capacity the same regardless of whether or not the cytosol contained only up-regulated (bound) receptors or a mixed population of bound and down-regulated unbound receptors. There was still a possibility that there may be some difference in the degree to which mercury will inactivate unoccupied up-regulated as opposed to unoccupied down-regulated receptors and this was examined in the following experiment. However, it is clear from the results of this experiment that the down-regulated receptor is not immune from the inactivating effects of mercuric chloride as was previously thought and is therefore less likely to be a useful tool in the determination of relative populations of up- and down-regulated receptors in situ.

Although previous experiments investigated the effects of the potentially irreversible sulfhydryl reactive reagents mercuric chloride, NEM and iodoacetamide on a standard cytosol preparation (containing a mixed population of up- and down-regulated DEX receptors) or, in the case of mercuric chloride, prelabeled receptors, the next experiment studied the effects of these reagents on receptor preparations containing either entirely up-regulated or down-regulated unoccupied receptors. The preparations were made by first preparing cytosol in the absence of DTT, then either adding DTT and incubating in ice for 6 hours (for up-regulated receptors) or not adding DTT and incubating at 22 C for 6 hours (for down-regulated receptors). Admitedly, this procedure may not have resulted in 100% down-regulation, but it appears to have been better than 90% effective. Both treatment groups were then subjected to Sephadex G-25 chromatography to remove DTT (where present) as well as endogneous small thiols that would interfere with the reaction prior to incubation with the reagent. Addition of excess DTT halted the reaction prior to steroid incubation. Consistent with the findings of the previous experiment, mercuric chloride incubation resulted in the same degree of inactivation of binding capacity for both up- and down-regulated groups (Figure 3-12). Both NEM and iodoacetamide significantly inactivated the binding capacity of the up-regulated group (about 90% binding loss), whereas they were differentially effective against the down-regulated group. Most of the down-regulated binding capacity was restorable after incubation with NEM (about 80% or better), while only about half was restorable after incubation with iodoacetamide. Since the ideal reagent would rapidly inactivate up-regulated receptors while remaining ineffective against

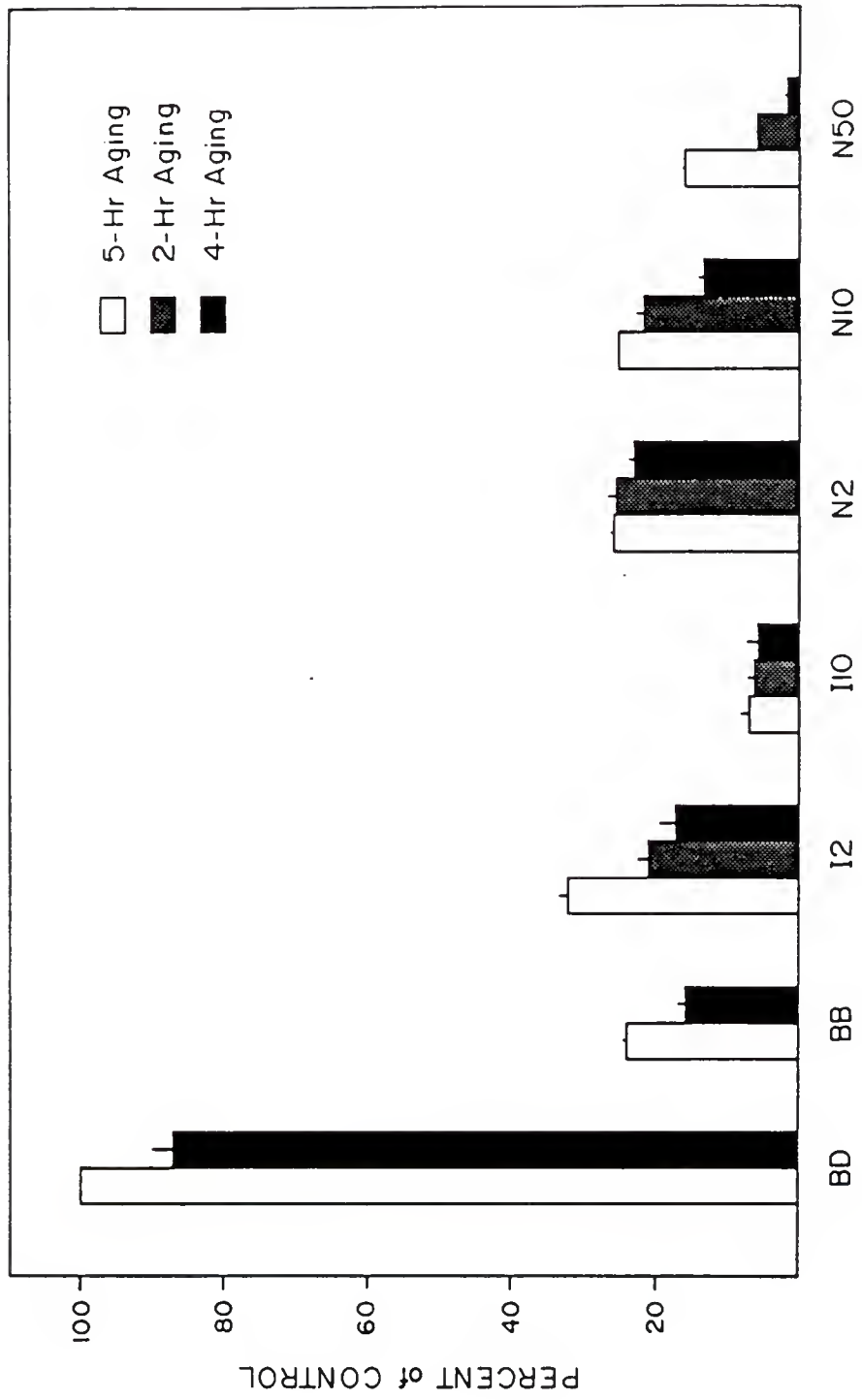
Figure 3-12. Effects of mercuric chloride, NEM and iodoacetamide on sulfhydryl up-regulated and down-regulated unoccupied Type II glucocorticoid receptors. Brain cytosol was prepared in HEPES buffer plus 25 mM molybdate and then either brought to 10 mM DTT and incubated at 0 C for 6 hr (up-regulated receptors, solid bars) or diluted with an appropriate volume of HEPES buffer only and then incubated at 22 C for 6 hr (down-regulated receptors, open bars). Each group was then run on Sephadex G-25 columns to remove DTT and/or other small thiol-containing molecules, followed by incubation with a 2 mM final concentration of mercuric chloride (Hg), n-ethyl maleimide (NEM) or iodoacetamine (IAM) for 1 hr at 0 C. Each group was then incubated with 20 nM [3H]DEX +/- 4 um [1H]DEX in the presence of 50 mM DTT for 40 hr at 0 C followed by bound-free steroid separations on Sephadex G-25 columns (open bars for B and DTT groups were incubated with steroid in the absence of additional DTT). Specific binding is expressed as percent of the DTT control group (open bar, 325 fmole/mg protein) and represents the mean +/- S.E.M. for 3 indepedent replications.


PERCENT of CONTROL
100
80
60
40
20
B
DTT
Hg
NEM
IAM

down-regulated forms, NEM appears to be the most appropriate reagent based upon these results. However, dose response and time course studies were required to more accurately characterize these reactions.

Time course and dose response studies of the effects of NEM and iodoacetamide on down-regulated and up-regulated unoccupied receptors (mixed population) were next performed in an effort to more clearly understand the effects of these two sulfhydryl reagents. Cytosol containing a mixed population of up- and down-regulated receptors was incubated at 0 C with 4 different concentrations (0, 2, 10 and 50 mM) of each of the two reagents for .5, 2 or 4 hours prior to the addition of excess DTT and incubation with [3H]DEX. Dose studies were necessary since it had not been determined how much of each of these reagents was required to saturate the endogenous thiols and other potentially reactive groups in crude cytosol (unchromatographed cytosol was used in this experiment for this reason). A time course was required to determine how rapidly the sulfhydryl oxidizing reaction occurs as well as if there are two or more reactions occurring at different rates (this might occur if the up-regulated form undergoes a rapid oxidation with a half-life of seconds or minutes and the down-regulated form undergoes a slow up-regulation and is subsequently oxidized quickly). Three replications of the experiment were performed with a high degree of replicability. As might have been expected from the previous experiment, NEM provided the most promising results (Figure 3-13) in that there appeared to be a "ceiling" effect with both time and dose (except for the highest dose). It is possible that a ceiling wasn't observed for the high dose because of a potentially inadequate concentration of DTT added to neutralize the reaction. With

Figure 3-13. Time course and dose response studies of the effects of NEM and iodoacetamide on a mixed population of sulfhydryl down-regulated and up-regulated unoccupied Type II glucocorticoid receptors. Brain cytosol was prepared in HEPES buffer plus 25 mM molybdate and incubated with a 0 (BD, BB), 2, 10 or 50 mM final concentration of either iodoacetamide (I) or n-ethyl maleimide (M) for 0.5 (open bars), 2 (screened bars) or 4 (solid bars) hr prior to the addition of 100 mM DTT (except for BB, which received an equivalent dilution with HEPES buffer only) and incubation with 20 nM [3H]DEX +/- 4 uM [1H]DEX at 0 C for 40 hr. Bound-free steroid separations were performed on Sephadex G-25 columns. Specific binding is expressed as percent of the BD, 0.5 hr, control group (360 fmole/mg protein) and represents the mean + /- S.E.M. of 3 independent replications.

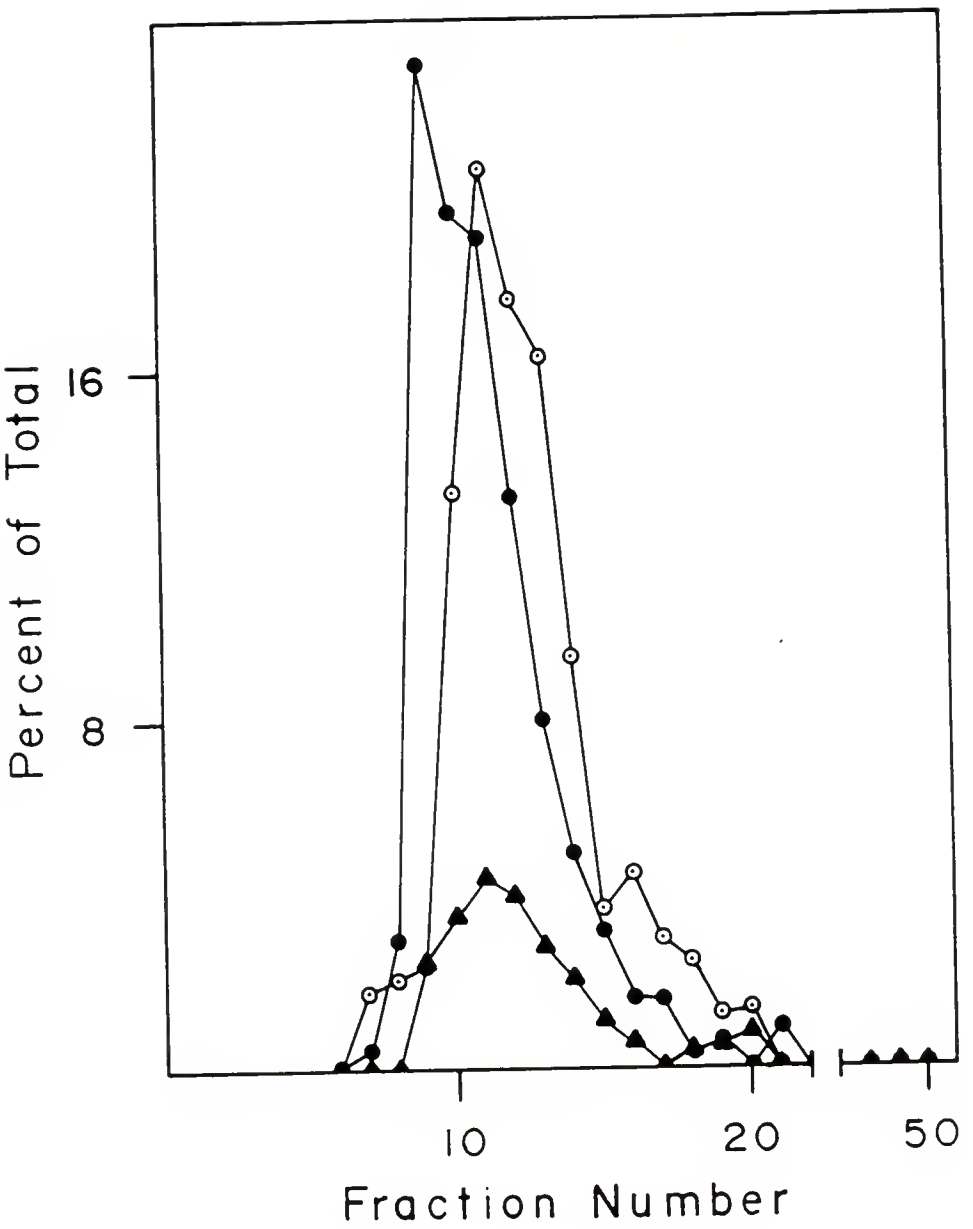

5-Hr Aging
2-Hr Aging
4-Hr Aging
PERCENT of CONTROL
100
80
60
40
20
BD
BB
I2
I10
N2
N10
N50

iodoacetamide, loss of binding capacity was time dependent regardless of concentration and higher concentrations resulted in total loss of all binding (including both up- and down-regulated forms). Perhaps iodoacetamide is less specific than NEM and may be inactivating down-regulated receptors by acting on nonbinding-site sulfhydryl or other reactive groups. Mercuric chloride was not examined in this experiment since the previous 2 experiments provided strong evidence that mercury does not distinguish between up- and down-regulated receptors.

Further attempts to characterize the differences in hydrodynamic properties that exist between the up- and down-regulated unoccupied glucocorticoid receptors included the hydrophobic interaction chromatography of both forms of the receptor on long pentyl agarose columns. Cytosol preparations that were either up- or down-regulated were equilibrated with HEPES buffer containing 600 mM KCl and 50 mM molybdate, but not DTT, prior to running on a pentyl agarose column equilibrated and eluted with the same buffer. Fractions were then incubated with 20 nM [3H]DEX +/- 4 uM [1H]DEX in the presence of 2 mM DTT. As shown in Figure 3-14, the down-regulated form appeared to have a slightly higher affinity for the hydrophobic matrix than the up-regulated form although the differences were not large.

The next experiment investigated the possibility that free thiol groups, other than those alledged to be in the steroid binding site, exist on the accessible surface of the glucocorticoid receptor. Cytosol labeled with [3H]TA for 40 hr at 0 C was chromatographed on each of several sulfhydryl affinity gels including activated thiol sepharose 4B, thiopropyl-sepharose 6B, glutathione disulfide agarose and sulfhydryl

Figure 3-14. Hydrophobic interaction chromatography of the sulfhydryl up-regulated and down-regulated Type II glucocorticoid receptors. Brain cytosol was prepared in HEPES buffer plus 25 mM molybdate and either brought to a concentration of 10 mM DTT and then incubated at 0 C for 8 hr (up-regulated receptor, open circles) or provided with an equivalent dilution of HEPES buffer only and incubated at 22 C for 8 hr (down-regulated receptor, solid circles). Each group was then run on a Sephadex G-25 column equilibrated and eluted with HEPES buffer plus 600 mM KCl and 50 mM molybdate. Aliquots (0.5 ml) from the resulting macromolecular fractions were run on long (7 ml) pentyl agarose columns, equilibrated and eluted with the same HEPES, KCl, molybdate, buffer and collected (0.5 ml/fraction) into tubes and placed on ice. Half of the contents of each tube was transfered to tubes containing 20 nM [3H]DEX and a sufficient concentration of DTT in HEPES buffer to result in a final concentration of 2 mM. The other half of each fraction was added to tubes containing the same concentration of labeled steroid and DTT plus 4 uM [1H]DEX for nonspecific binding profile determination (solid triangles, same profile results for both groups). Each condition was run in triplicate and the profiles shown are representative.


Percent of Total
16
8
10
20
50
Fraction Number

cellulose. The sulfhydryl reagents covalently linked to the gel matrices react to varying degrees with the accessible free thiols of cytosolic proteins resulting in mixed disulfide formation and the retention of the thiol-containing protein by the gel. Since the thiol group(s) whose status determines the receptor's ability to bind steroid ligand is supossedly protected by the presence of a bound ligand (as indicated by the results of the previous experiment), the retention of bound glucocorticoid receptors by sulfhydryl affinity gels would indicate the likely presence of additional surface thiol groups. The existence of such extra-binding site thiols could affect the interpretation of quantitative sulfhydryl studies as well as allow for partial purification of the bound receptor. Varying degrees of retention of the [3H]TA-labeled receptors were displayed by the gels with thiopropyl-sepharose 6B having the highest affinity for receptors and thiol sepharose 4B exhibiting the lowest affinity (data not shown). This step was followed by an elution with DTT-containing buffer which reduces the mixed disulfide bonds, thereby freeing the receptor from the gel matrix and allowing for its removal from the column. The receptor elution profiles after DTT elution again varied depending upon the gel, but nevertheless providing unquestionable evidence for additional surface free thiols.

Discussion

Since sulfhydryl group oxidation was implicated in the inactivation of estrogen receptor binding (Jensen et al., 1967), the phenomenon has been reported for practically all other steroid receptor types including glucocorticoid receptors. Unfortunately, sulfhydryl oxidation and reduction of steroid receptors has been viewed by most workers in the

field as merely an in vitro inconvenience to be overcome by including sulfhydryl reducing reagents, such as DTT or mercaptoethanol, in their buffers. Few viewed the process as a potential mechanism by which preexisting receptor binding sites were up- or down- regulated in vivo and, as a result, many aspects of glucocorticoid receptor sulfhydryl oxidation/reduction have not been thoroughly investigated. This study sought to examine the process in much greater detail than had been previously reported in an attempt to determine if it was even feasible to suggest such an in vivo role for oxidation/reduction in the regulation of glucocorticoid binding capacity. The initial experiments in this study confirmed in brain, liver and kidney cytosol what had previously been reported regarding the requirement for sulfhydryl reducing reagents in order to optimize glucocorticoid receptor binding in thymus cytosol and that there is a temperature-dependent decline in this binding in the absence of such reagents (Rees and Bell, 1975). The results obtained here for brain and other tissues also confirmed the findings of Sando et al. (1979) for thymic lymphocyte cytosol, who reported a complete restoration of this lost binding capacity upon readdition of DTT if molybdate had been present in the buffers. A restoration of binding upon DTT addition had been reported earlier by Granberg and Ballard (1977) for cytosol from several tissues, including brain, but this restoration was only partial because of the lack of molybdate in their buffers.

The fact that nearly 30 hr were required in order to completely down-regulate glucocorticoid binding capacity in brain cytosol may not be very relevant, in a physiological sense, to the understanding of down-regulation in vivo, if, in fact, it does occur there. However, the

fact that binding capacity could eventually be reversibly reduced to nothing has important implications for the understanding of how sulfhydryl oxidation interferes with measured glucocorticoid binding capacity. Another potentially important finding during these initial experiments was the fact that unoccupied receptors in the down-regulated or oxidized state do not appear to be any more or less susceptible to irreversible inactivation than those receptors in the up-regulated or reduced state. It is known that a number of biochemical processes involving proteins, such as phosphorylation (Chen and Kim, 1985), are dependent on the sulfhydryl-disulfide state of the protein. It is possible, however, that the presence of molybdate may have prevented any increase in the rate of irreversible inactivation induced by either sulfhydryl oxidation or reduction.

In order to gain a better understanding of the nature of the factors in cytosol responsible for maintaining the receptor in an up-regulated state, cytosol was pretreated with dextran-coated charcoal and the rate of reversible binding loss was evaluated at elevated temperatures as it was in previous experiments. It is clear that an endogenous factor responsible for the up-regulation of unoccupied receptors is removed by the charcoal treatment, since the rate of sulfhydryl down-regulation is dramatically increased. One must, however, consider the possibility that charcoal is removing a factor which indirectly affects the up-regulation of receptors. According to Gilbert (1984), for thiol/disulfide exchange to be considered as a feasible biological control mechanism for protein action (enzyme activity, receptor binding, etc.), the thiol/disulfide redox potential of a regulated protein should be near the observed thiol/disulfide ratio

in vivo. Since reduced thiols are far more reactive than disulfides, the charcoal treatment is likely to have removed a proportionately larger amount of the free thiol-containing molecules than the disulfide-containing molecules, leading to a potentially significant shift in the overall thiol/disulfide ratio. One piece of evidence that charcoal is removing small molecular weight thiols comes from the finding in this study that charcoal treatment of cytosol already containing DTT still results in a loss of binding capacity that is reversible upon readdition of DTT after charcoal treatment. Interestingly, the binding loss due to charcoal treatment of pre-DTT-treated cytosol was not fully reversible in brain cytosol, whereas it was fully reversible in liver and kidney cytosol when DTT was readded. One possible explanation is that pretreatment with DTT, by reducing not only the sulfhydryl(s) involved with steroid binding but also other sulfhydryls, probably on the receptor's surface (Bodwell et al., 1984b), increased the likelihood that some of the receptors would themselves be adsorbed by the activated charcoal. The probability of this occurring in liver or kidney cytosol was probably minimized by the fact that these cytosols had a higher protein concentration.

It was discovered from these same experiments that when the charcoal treatment was omitted, cytosol from both liver and kidney exhibited a much greater loss of binding due to sulfhydryl oxidation (resulting from omission of DTT) than was observed for brain cytosol. This is in direct opposition to the findings of others, particularly Granberg and Ballard (1977), who reported that binding activity in both liver and kidney cytosol in the absence of DTT was 100% of the level measured in the presence of DTT, whereas binding activity for brain

cytosol in the absence of DTT was approximately 61% of that measured in the presence of DTT, a level similar to our own findings for brain. These workers suggested that the lack of a DTT requirement in liver may have been, in part, due to the high level of "endogenous reducing agents" and indicated that the levels of non-protein sulfhydryl groups were approximately 6-fold greater than they were in brain cytosol. On the other hand, the levels for non-protein sulfhydryl groups in kidney cytosol were reportedly identical to those in brain cytosol, leaving the impression that knowledge of sulfhydryl group levels alone, without knowledge of disulfide levels, may be insufficient to predict the relative binding levels in the absence of exogenously added sulfhydryl reagents. The fact that the two studies differed so widely in their findings regarding the DTT requirement for maximal glucocorticoid binding in liver and kidney cytosols may be an indication of the sensitivity of this process to variation in experimental procedure. Since binding capacity cannot be determined instantaneously, the duration and temperature of steroid incubation as well as buffer composition, pH, steroid concentration, etc. will almost certainly affect the outcome of this type of experiment.

The inactivating effects of charcoal treatment on glucocorticoid receptor binding capacity and the ability of various factors to reverse these effects have been reported on by Grippo et al. (1983), who also found that lost binding capacity was restored by adding DTT. These workers also reported that incubating charcoal-treated cytosol with boiled liver cytosol partially restored lost binding capacity. Two components of the boiled cytosol were found to be required for restoration of charcoal-induced losses in receptor binding capacity: NADPH and

an endogenous heat-stable protein later "proven" to be thioredoxin (Grippo et al., 1985). Though they claimed to have presented proof that the endogenous heat-stable factor was, in fact, thioredoxin, their research does not completely rule out the possibility that other charcoal-extractable, heat-labile factors may also play an important role in direct or indirect glucocorticoid receptor sulfhydryl reduction. One enzyme, designated thiol:protein-disulfide oxidoreductase, catalyzes the reductive cleavage of insulin and is also heat-stable, but requires reduced glutathione, or possibly other small thiols that are also extracted by charcoal, as a cosubstrate (Bjelland et al., 1984).

The fact that antibodies against thioredoxin reductase inhibit up regulation of glucocorticoid receptor binding capacity in charcoal-treated cytosol by boiled liver cytosol has been used by these workers as the "proof" that the endogenous heat stable glucocorticoid receptor up-regulating factor is thioredoxin. However, one of the coauthors of the report had previously shown thioredoxin reductase from mammalian tissues to have a fairly wide substrate specificity (Holmgren, 1977). Although Holmgren (1977) claimed that in a purified system, disulfide bond reduction in insulin or L-cystine by NADPH and thioredoxin reductase was dependent upon thioredoxin as an intermediate electron carrier, the same study indicated that the enzyme could reduce other potentially relevant endogenous compounds, such as the oxidized forms of glutathione and lipoic acid as well as the artificial substrate DTNB "directly without thioredoxin". Lipoamide dehydrogenase and glutathione reductase are both capable of catalyzing net reduction of disulfides. Glutathione reductase catalyzes the reduction of oxidized glutathione and, like thioredoxin reductase, also requires NADPH.

Reduced glutathione, in turn, can reduce a wide variety of disulfides by transhydrogenation. The later reactions are catalyzed by thiol-transferases (Mannervik and Axelsson, 1975) and glutaredoxin (Luthman and Holmgren, 1982), both of which are cytosolic and are similar in molecular weight to each other (about 11,000) as well as to thioredoxin (about 12,000). Although the substrate specificities of these enzymes, like thioredoxin reductase, are rather broad, thiol transferase catalyzes transhydrogenation of a larger variety of different thiols and disulfides as well as reversible formation of protein-mixed disulfides (Axelsson and Mannervik, 1980). One point in favor of thioredoxin being of significant importance in the maintenence of the up-regulated form of the unoccupied glucocorticoid receptor comes from a comparison of the reductive capacity of the thioredoxin system in rat liver with that of the glutathione-thiol transferase system (Mannervik et al., 1983), wherein it was shown that while the thiol transferase system is more efficient in catalyzing reduction of small disulfides, thioredoxin was slightly more effective in reducing exposed disulfides in peptides.

Since the discussion thus far has focussed on enzymes and small molecules potentially involved in the reduction or up-regulation of glucocorticoid receptors, it is important to consider, momentarily, those endogenous factors that might be involved with thiol oxidation. This cannot be overlooked if sulfhydryl down-regulation of glucocorticoid receptor binding capacity is to be considered an active physiological process. Although nonenzymatic oxidation of thiols within intact cells is probably insignificant (Ziegler, 1985), enzyme-catalyzed reactions capable of net generation of disulfides within cells are quite limited and only a few have been well characterized. The oxidation of

glutathione by H202, catalyzed by glutathione peroxidase, is usually considered the major route for enzymatic generation of cellular disulfide. However, additional intracellular disulfides are contributed as the result of the desulfuration of 3-mercaptopyruvate, catalyzed by the mercaptopyruvate transsulfurase, and to a lesser extent by the oxidation of cysteamine to cystamine, catalyzed by the membrane-bound flavin-containing monooxygenase (Ziegler, 1985). A fact relevant to the interpretation of experiments regarding sulfhydryl down-regulation of binding capacity over time in cytosolic preparations is that much of the enzyme activity associated with sulfhydryl oxidation/reduction is either membrane associated or extracellular. Most, if not all, of the membrane-associated activity will be absent from cytosol, and therefore not contribute to sulfhydryl oxidation after cytosol preparation is complete. However, some of the membrane associated enzymes, such as thiol oxidase (Ziegler, 1985), apparently have their active sites directed extracellularly, where the thiol:disulfide ratio is dramatically (50- to 100-fold) lower. These enzymes would normally not influence the glucocorticoid receptor binding capacity in vivo since these receptors are intracellular. However, tissue homogenization could potentially lead to an increase in enzyme-induced receptor oxidation during cytosol preparation in the absence of excess sulfhydryl reducing agents. In addition, the overall thiol/disulfide ratio of the homogenate and, subsequently, the resulting cytosol will be much lower and the potential for sulfhydryl oxidation much higher as a consequence of the contribution of high disulfide concentration and soluble extracellular nonspecific thiol oxidases characteristic of extracellular fluids.

Finally, Grippo et al. (1985) have provided no evidence as to whether thioredoxin simply reduces a disulfide bond to yield a reduced, steroid binding form of the glucocorticoid receptor or whether, as in the case of T7 DNA polymerase (Mark and Richardson, 1976), reduced thioredoxin must be bound to the receptor for it to be in a steroid binding conformation. The possibility that charcoal treatment may have an affect directly on the glucocorticoid receptor itself has been proposed by Bell et al. (1984) who reported that charcoal pretreatment of unlabeled receptors led to a significant reduction in steroid dissociation rate after steroid labeling and suggested that this change was a consequence of the removal of a lipid component from the complex. Although these authors don't implicate sulfhydryl oxidation/reduction in the reduced steroid dissociation rates, it has been reported by Takabayashi et al. (1983) that the binding of fatty acids to bovine serum albumen increases the rate of oxidation of a free sulfhydryl group, while the oxidation of the sulfhydryl enhances the binding of fatty acids to the protein. Fishman (1983) reported similar effects of charcoal treatment on estrogen receptors when he showed that treating uterine cytosol with dextran-coated charcoal in the absence of ligand causes the subsequently formed receptor-estradiol complex to be stable at 37 C, although again, the results were too inconclusive to implicate sulfhydryl oxidation/reduction.

The effect of sulfhydryl up- and down-regulation on the structure of the unoccupied glucocorticoid receptor has never before been investigated. Although the characterization of nonsteroid-labeled receptors entails a number of obvious difficulties, such an investigation might provide valuable clues as to whether or not

sulfhydryl up- and down-regulation involves merely a thiol/disulfide exchange reaction within the receptor or mixed disulfide formation with another small molecule, peptide, protein, etc. as was suggested by Grippo et al. (1985) as a possible means of thioredoxin interaction with the glucocorticoid receptor. In addition, even if the oxidation reaction is completely internal (inter- or intra-subunit disulfide formation), significant conformational changes are possible which could lead to measurable changes in sedimentation coefficient, hydrophobicity, etc. One experiment in this study provided evidence that whatever changes that occur during sulfhydryl oxidation/reduction do not affect the receptor conformation or overall molecular mass to an extent detectable by changes in sedimentation coefficient. Because of the relative insensitivity of such a measurement, one cannot rule out the possibility of mixed disulfide formation with a small molecule such as glutathione, and it is conceivable that simultaneous changes in molecular mass and conformation could result in a new form with sedimentation characteristics very similar to the original form. However, the sedimentation results indicate that at least no major changes are likely. Although Wilson et al. (1986) recently reported changes in sedimentation properties of the androgen receptor associated with sulfhydryl oxidation and reduction, these changes occurred in the absence of molybdate and presumably were linked to the process of receptor activation.

Possible changes in hydrophobicity associated with sulfhydryl up- and down-regulation were also investigated using pentyl agarose hydrophobic interaction chromatography. Again, no significant changes were detected. This is somewhat of a surprize since it is well known

that reduced proteins are generally less soluble and have a strong tendency to aggregate (Gilbert, 1984). In addition, the oxidation of a thiol that presumably resides in or near the hydrophobic steroid binding site of the receptor might be expected to interfere to some extent with hydrophobic interactions. It must again be emphasized, however, that the nature of these experiments and the lability of the unoccupied glucocorticoid receptor under such conditions necessitated the use of molybdate in most buffers. Although the molecular mechanism by which molybdate stabilizes steroid receptors is not clearly known, it is possible that molybdate may be interacting to form bridge structures between adjacent sulfhydryl groups (Wilson, 1986). Molybdate, therefore, might influence otherwise detectable sulfhydryl oxidation/reduction-mediated changes in receptor properties.

Scatchard analysis of [3H]DEX binding to cytosol in the presence or absence of DTT led to the impression that not only did DTT increase the maximum binding capacity, but also the apparent affinity of the receptor for DEX. Because of the potential implications of such a finding for the role of sulfhydryl oxidation/reduction in steroid binding and, consequently, steroid action, this phenomenon was further investigated. Kinetic studies showed that neither the presence or absence of DTT had any effect on the rate of dissociation of [3H]DEX from the prelabeled receptor. These results differ from those reported in a similar study on the effects of DTT on DEX dissociation kinetics, in which case DTT was found to increase the rate of dissociation under certain conditions, while it was reportedly ineffective under other conditions (Buell et al., 1986). Unfortunately, this study was flawed in that 12 mM monothioglycerol was present in all buffers, even in the nonDTT

experimental groups. It is puzzleing why these workers included monothioglycerol in their study since it is a sulfhydryl reducing agent and, by their own admission, has been shown to have "an effect on binding similar to DTT". One might argue then, that the appropriate controls were absent from this study. In addition, these workers found that dissociation rate was not affected by DTT if removal of free DEX was initiated by a dextran-coated charcoal treatment (without a subsequent readdition of DTT). Our own work and that of others (Grippo et al., 1983, 1985) has shown that charcoal treatment removes much, if not all, of the reducing capacity of cytosol, including exogenously added DTT as well as endogenous reducing factors. Buell et al. (1986) did observe an increased dissociation rate in the presence of DTT if dissociation was initiated by addition of a 1000-fold concentration of either unlabeled DEX or unlabeled RU5020, a synthetic progestin shown to facilitate glucocorticoid dissociation. A potentially important difference between our study and that of Buell et al. (1986) was that our own work used a DTT concentration of only 2 mM, shown in a number of experiments in this study to be more than sufficient to up-regulate all glucocorticoid binding capacity, whereas the other workers used 20 mM DTT. It is possible that if the DTT effect reported by these workers is real, it may be concentration dependent and may involve effects on glucocorticoid receptors very different from the reduction of the binding site sulhydryl group(s). These researchers reported earlier in abstract form on the effect of DTT on the rate of association of [3H]DEX to glucocorticoid receptors (Buell et al., 1986). They claimed that DTT led to a slight decrease in the association rate constant. Results from our own efforts to determine effects of DTT on association rate were

deemed uniterpretaable because of the fact that unoccupied receptors in cytosol not containing DTT are undergoing down regulation for the duration of time that the association rate is being determined, thereby significantly complicating the determination of rate constants.

In light of the findings of Buell et al. (1986), wherein DTT increased dissociation rate and decreased association rate, one would assume that Scatchard analysis of DEX binding in the presence and absence of DTT would result in an apparent reduction in affinity for cytosol containing DTT. However, as previously stated, our own work showed the opposite to be the case in that DTT led instead to an apparent increase in affinity for DEX binding. It is now thought that this apparent change in affinity can be explained, for the most part, by the selective down regulation of the unoccupied, but not the occupied, form of the receptor. Factors that can differentially affect the stability of the bound and unbound forms of the glucocorticoid receptor (or any other type of receptor for that matter) can potentially result in artefactual changes in various binding parameters as determined by Scatchard analysis as has been shown by computer simulation (Beck and Goren, 1983; Ketelslegers et al., 1984). Since Scatchard analysis involves the incubation of identical cytosols with varying concentrations of free ligand, receptors incubated with relatively small concentrations of steroid will, on average, be in an unbound form for a much greater percentage of the incubation period, and, thus, be more susceptible to the types of inactivation that are inhibited by the binding of a steroid ligand, including sulfhydryl down-regulation. Therefore binding in these samples will appear artefactually small. However, receptors in samples incubated with steroid concentrations in

ess of saturation levels will remain unbound for a relatively short iod of time, resulting in little appreciable loss of binding capacity ing the incubation period. If such a situation is true, changes in ubation time and temperature will have additional influence on the ree of artefactual change observed in these types of determinations. course, these artefactual changes would not occur in the presence of since both occupied and unoccupied forms of the receptor appear to equally stable under the experimental conditions since sulfhydryl n-regulation is no longer a factor.

The next phase of this study, that dealing with the effects of ous reversible and irreversible sulfhydryl reactive reagents on ocorticoid receptor down-regulation was carried out with several s in mind. The first of these was to determine how effective these erent reagents were in down-regulating receptors under the buffer itions used. Most sulfhydryl reactions are very pH dependent, gh they are much less affected by temperature. In addition, bility, reactivity with other buffer components and the ability of effects of the reagent to be neutralized by DTT all had to be stigated early on. Of major concern was whether the loss of ocorticoid binding capacity attributed to a particular reagent was rsible upon addition of excess DTT and to what extent. The ficity of the reaction against up-regulated or down-regulated upied receptors or even occupied forms of the receptor was also cally important. The effectiveness of different reagents might de some indication of the nature of the sulfhydryl group(s) being zed during down-regulation. An excellent example of this was ted by Formstecher et al. (1984) who determined the

pseudo-first—order kinetics for the irreversible inactivation of glucocorticoid receptor binding capacity in liver using a series of n-alkylmaleimides. These workers demonstrated a striking increase of receptor inactivation with increasing chain length of the maleimide derivative while steroid binding continued to afford full protection against inactivation. It was suggested that the chain length effect observed in the inactivation process was related to nonpolar interactions in the binding of maleimides to the receptor prior to the irreversible alkylation of sulfhydryl groups. Because of the hydrophobic nature of the environment where these sulfhydryl groups were located, the authors were able to conclude that these groups were probably in the binding site itself. Because one of the goals of the present study was to determine, if possible, the in vivo ratios of up- and down-regulated forms of the glucocorticoid receptor, most of the experiments were performed using either crude cytosol or crude homogenate or, in some cases, a partially purified preparation. The kind of conclusions drawn by Formstecher et al. (1984) regarding the molecular localization of the sulfhydryl group(s) critical to steroid binding required that at least a partially purified system be used. This was in large part because the pseudo-first-order kinetics of receptor inactivation would be difficult to obtain in the presence of high concentrations of free thiol groups, particularly in liver. This brings up several problems relevant to not only sulfhydryl studies of steroid receptor binding capacity, but to sulfhydryl studies in impure systems in general. First, as stated previously, numerous enzymes (both intra- and extracellular in origin) with broad substrate specificities can be found in cytosol preparations. Secondly, small thiol and

disulfide "contaminants", again introduced by mixing of intra- and extracellular fluids during homogenization, can dramatically alter the thiol/disulfide status relative to normal intracellular levels. Thirdly, air oxidation of free thiols, though generally a slow process, can be a greater problem for a particular protein thiol in an impure system (because of indirect oxidation) than in a pure system. Finally, most sulfhydryl reactive reagents are relatively nonspecific and modification of one particular sulfhydryl group in a system is generally accompanied by a similar modification of many, if not most, of the other sulfhydryls in the system. This, of course, often perturbs the entire thiol/disulfide balance for the system which, in turn, may have consequences for those protein sulfhydryls or disulfides that aren't directly impacted by the reagent in question, further complicating the interpretation of results.

Although many of the experiments in the present study indicated a relatively slow rate of down-regulation in cytosol at low temperatures, the situation for receptors in homogenate, for the reasons already discussed, appears to be more complex. For this reason the determination of in vivo levels of up- and down-regulated receptors would require that the two forms of the receptor be rapidly trapped in their particular sulfhydryl configuration either during or immediately after tissue homogenization. Initial experiments in this series, performed on cytosolic preparations, demonstrated that some reagents, such as DTNB, cystamine, glutathione disulfide, DTNT and MMTS were capable of down-regulating glucocorticoid receptors in a fashion that was completely reversible with DTT. A potentially more valuable finding, however, was that receptors down-regulated with some of these reversible reagents

appeared to be almost completely protected from irreversible inactivation by some of the irreversible sulfhydryl reactive reagents. If the endogenously down-regulated form of the receptor possesed the same resistance to irreversible inactivation by NEM or iodoacetamide, then it could later be up-regulated to an assayable steroid binding form by addition of [3H]DEX and an excess of DTT, which would simultaneously neutralize the inactivating reagent. The original population of up-regulated receptors could be determined indirectly by measuring total binding capacity in the presence of DTT, but with no inactivation treatment, and then subtracting the down-regulated level. This, in fact, was the goal for the experiments that followed. However, the matter proved to be far more complicated than originally thought, and the chemical reactions involved with this "trapping" procedure had to be further investigated and characterized in detail.

The potential up regulation of down-regulated receptors during the incubation of homogenate or cytosol with sulfhydryl inactivating reagents was another problem that had to be considered since the inactivation reaction was not instantaneous. This is likely to be a major problem only if the inactivation reaction is slow. Otherwise, the small thiols (such as glutathione) necessary as electron acceptors for reduction of down-regulated receptors would themselves be consumed by the reaction, thereby shifting the reaction in favor of receptor oxidation. The rate of the inactivation reaction was therefore of serious importance. Since the rate of such reactions depends most heavily on the concentrations of the reactants and since the binding site sulfhydryl group(s) of the up-regulated receptor is competing with a many thousand-fold greater concentration of free sulfhydryl groups in

cytosol or homogenate, a relatively high concentration of either NEM or iodoacetamide was required to achieve acceptably fast inactivation. Unfortunately, raising the concentration of these reagents presented yet another problem in that high concentrations of iodoacetamide has been shown to produce modifications of other protein groups. Although it isn't known for sure how such non-sulfhydryl modifications of proteins attributed to iodoacetamide could lead to inactivation of the normally-protected down-regulated glucocorticoid receptor, but results from several experiments in the present study indicated that higher levels of iodoacetamide eliminated all of the binding capacity in cytosol known to contain down-regulated receptors. Like iodoacetamide, reaction of NEM with proteins is not completely specific for sulfhydryl groups, but also occurs with imidazole and alpha-amino groups via a similar mechanism. However, reaction with sulfhydryl groups occurs much faster, and the rate of the reaction with sulfhydryls is markedly enhanced with increasing pH. At pH 7, the reaction rate of NEM with simple thiols is approximately 1000-fold greater than with corresponding simple amino compounds. Therefore, at the pH of 7.6 used in the present study, NEM reaction with amino groups does not appear to have been a problem.

Another related problem that had to be dealt with simultaneously involves the effects of high concentrations of DTT on glucocorticoid receptor binding capacity in tissue homogenate. When high concentrations of either iodoacetamide or NEM are used, even higher concentrations of DTT are required to neutralize the reaction and up-regulate the down-regulated forms. It was found in the present study that concentrations of 50 mM and higher DTT, when added to homogenate,

inactivated glucocorticoid receptor binding, even in the absence of the other inactivating reagents. Even though DTT has a similar effect on binding when added to cytosol, much higher concentrations are required. The mechanism of this DTT-induced inactivation is unknown, although it is possible that protein aggregation was enhanced by these high concentrations of reductant. If this is the case, aggregation would expect to be even more of a problem in a homogenate suspension than in cytosol. This unexpected problem placed even further restrictions on inactivating agent concentration.

CHAPTER IV

THE PURIFICATION AND SUBSEQUENT ACTIVATION OF THE GLUCOCORTICOID RECEPTOR

Introduction

Purification

A major problem in interpreting much of the published work concerning the physicochemical properties of glucocorticoid (and other steroid) receptors is that all but the most recent studies were conducted on either crude or only partially purified receptors. In light of the numerous demonstrations that various endogenous "factors" and exogenously added substances can dramatically affect receptor stability, binding and activation kinetics, specificity and affinity of nucleic acid binding, the purification of the receptor is clearly an essential next step in many of these studies. A number of approaches have been taken to achieve varying degrees of purification of glucocorticoid and other steroid receptors. The strategy of choice depends on the form of receptor desired (unoccupied, occupied-unactivated or occupied-activated), the degree of purification required for a given procedure and various other limitations or requirements. A combination of different purification steps will likely be required to produce optimal results in most cases.

Any attempt at glucocorticoid receptor purification, however, must take into account the following difficulties: (1) limited sources of a tissue abundant in free receptor, (2) the presence of proteins other

than the receptor which bind to glucocorticoids, (3) the high degree of purification necessary to obtain homogeneous preparations and (4) an apparent instability of all forms of the receptor (Santi et al., 1979). The last difficulty, receptor instability, was a major factor limiting many early attempts at receptor purification, particularly in light of the fact that the apparent instability of the receptor from some sources increases as the receptor is purified (perhaps due to the removal of endogenous stabilizing factors). However, the findings of Leach et al. (1979) demonstrating the stabilization of each of the glucocorticoid receptor forms by molybdate greatly diminished the limitations placed on purification by receptor instability. Of course, the fact that the precise nature of the mechanism by which molybdate (and other transition state elements) stabilizes the glucocorticoid receptor remains unknown should not be overlooked when interpretting the results of experiments performed in the presence of molybdate.

Earlier attempts to purify the glucocorticoid receptor generally involved a series of chromatographic steps typically including ion-exchange, gel exclusion and DNA-cellulose (Santi et al., 1979; Govindan and Manz, 1980; Romanov and Gorshkova, 1984). The degree of purification, as well as the yield, varied considerably from study to study, although impressive results were recently obtained by Govindan and Gronemeyer (1984) and Moudgil et al. (1985) using a refined DNA-cellulose procedure and by Wrange et al. (1984) using a sequential chromatography on DNA-cellulose and DEAE-Sepharose. The use of steroid affinity resins has increased markedly the degree of purification attainable in recent studies. Deoxycorticosterone hemisuccinate coupled covalently to BSA-Sepharose 4B was used to achieve a relatively low

level of purification of glucocorticoid receptors from rat brain (De Kloet and Burbach, 1978). More recently, deoxycorticosterone-agarose has been used for the purification of both progesterone (Grandics et al., 1982) and glucocorticoid (Grandics and Litwack, 1982) receptors. Basically, cytosol is applied to the affinity gel and binding to the deoxycorticosterone is allowed to reach equilibrium. The gel is then washed free of nonbinding components and is incubated with high concentrations of the free tritiated steroid of choice. The highly specific synthetic [3H]triamcinolone acetonide was used for the exchange assay for glucocorticoid receptors since it binds neither progesterone nor mineralocorticoid receptors nor corticosteroid binding globulin (CBG), which are all likely to bind to some extent to the deoxycorticosterone resin. After the exchange reaction has gone to (or near) completion, the radiolabeled receptors can simply be washed from the resin and further purification steps can be applied as necessary. Very recently a 4000-fold purification of rat liver glucocorticoid receptor was achieved using this procedure (Grandics et al., 1984b). Govindan and Manz (1980) and Lustenberger et al. (1981) achieved similar results using an affinity column containing either dexamethasone-17B-carboxylic acid or dexamethasone-21-methanesulfonate coupled to an aminocystamido-succinylamidohexyl-Cl-Sepharose 4B column. Govindan and Gronemeyer (1984), using this same ligand affinity procedure, have recently increased the yield of purified receptor significantly. Other procedures using affinity resins for the purpose of purification have included the binding of glucocorticoid-biotin analogs to the receptor prior to incubation with an avidin-Sepharose affinity gel (Manz et al., 1983). These workers reported a 19,000-fold purification of the

glucocorticoid receptor from human spleen tumor cytosol. Other steroid receptor systems have been purified by various types of affinity chromatography. Progesterone receptor from chick oviduct has been purified to a high degree (1500-2700-fold) using a N-(12-amino-dodecyl)-3-oxo-4-androsten-17B-carboxamide-substituted Sepharose gel (Renoir et al., 1982). As with glucocorticoid receptors, combining this affinity chromatography step with an ion-exchange chromatography step significantly increased the degree of purification (to >6700-fold). Smith et al. (1981) reported a 67,000-fold purification of human uterine progesterone receptor using a combination of ammonium sulfate fractionation and affinity chromatography (deoxycorticosterone-BSA-Sepharose 4B). More recently, Van Oosbree et al. (1984) used diethylstilbestrol coupled to epoxy-activated agarose to yield highly purified rabbit uterine estrogen receptors upon elution with p-sec-amylphenol and NaSCN (a chaotropic salt). More recently, amino-aryl controlled pore glass beads coupled to a variety of steroids (including several glucocorticoids) using a bifunctional cross-linker with amino reacting and photogenerated nitrene functions (Lingwood, 1984) have become commercially available. However, no reports have yet appeared concerning the use of these steroids immobilized to glass beads for steroid receptor purification purposes. Krajcsi and Aranyi (1986) most recently reported the use of cortexalone-Sepharose for the purification of thymus glucocorticoid receptors. This new affinity matrix only resulted in a 75-150-fold purification with a yield of 20-30%, but the relatively fast dissociation of the glucocorticoid receptor-cortexalone complex allows for a much more rapid purification step.

Another form of affinity chromatography that has been reportedly used for the purification of estrogen and glucocorticoid (Weisz et al., 1984) receptors involves the use of immobilized heparin. However, heparin has been shown to cause inactivation of unoccupied progesterone (Thorsen, 1981) and glucocorticoid (Hubbard and Kalimi, 1983; McBlain and Shyamala, 1984; Densmore et al., unpublished results) receptors as well as activation of bound receptors (Thorsen, 1981; Yang et al., 1982; Hubbard and Kalimi, 1983c; McBlain and Shyamala, 1984). In addition, the degree of purification obtained with heparin chromatography (only about 10-fold (Weisz et al., 1984)) is exceptionally low and would best be used for nonquantitative characterization or receptor activation studies (Blanchardie et al., 1984). More recently Weisz et al. (1986) used Sepharose-heparin to partially purify Type I, or mineralocorticoid, binding activity of kidney cytosol. Although a yield of around 90% was reported and all CBG was removed by the process, the degree of purification was once again in the range of only 10-fold.

Finally, immunopurification was recently described as a means of purifying [3H]dexamethasone mesylate-labeled receptors (Smith and Harmon, 1985). Labeled cytosol was first incubated with anti-glucocorticoid receptor serum followed by adsorption of the antibody-receptor complexes to protein A immobilized to Sepharose CL-4B. These workers did not report the degree of purification or the yield from this procedure. Although this study used prelabeled receptors, the technique offers a means of purifying nonsteroid-labeled glucocorticoid receptors.

Activation

Probably the most accepted definition of "activation" is simply the process whereby the steroid-receptor complex is converted to a form able to bind to its nuclear acceptor sites. Precisely what physicochemical changes occur in the receptor molecule during this process, what endogenous factors, if any, regulate this process and what, exactly, constitutes an "acceptor" site remains controversial. However, despite the lack of total agreement on these matters, certain changes in the basic properties of the glucocorticoid-receptor complex during activation are now generally accepted: 1) an increase in the affinity of the receptor complex for natural and synthetic polyanions (apparently by virtue of an increase in surface positivity), 2) a decrease in the overall size of the receptor complex and 3) a decrease in the dissociation rate constant for the receptor complex (although this now appears to depend upon the ligand bound). Thus, the most obvious means by which the process of activation could be qualitatively and quantitatively studied would involve the separation of the activated and unactivated forms of the receptor complex based on their size and/or surface charge differences. A variety of chromatographic and other techniques have been used to study some of the physicochemical changes in the glucocorticoid-receptor complex which accompany activation. However, much of the results remains controversial due to a failure to control for receptor lability and activation during the long procedures required for determination of these physiochemical parameters. As mentioned previously, the discovery that molybdate prevents both the loss of unoccupied receptors and the activation of occupied receptors (Leach et al., 1979) has removed many of these difficulties and has allowed a much

greater resolution of unactivated from activated glucocorticoid-receptor complexes (Luttge and Densmore, 1984; Luttge et al., 1984a,b,d). The following section will briefly review some of the more promising techniques of activation analysis which now demand renewed attention in light of recent developments in receptor stabilization and purification.

Historically, it was the observation that glucocorticoid-receptor complexes translocated and bound to the nuclei of glucocorticoid target cells that provided the most convincing evidence of their genomic mechanism of action. Early studies by several laboratories demonstrated that when nuclei isolated from hepatoma cells in tissue culture or liver cells were incubated with the synthetic glucocorticoid [3H]dexamethasone, very low levels of nuclear binding were detected. However, if the steroid was first equilibrated with cytosol at 0 C and this mixture was then incubated with nuclei at 20 C, time-dependent specific nuclear binding of the tritiated glucocorticoid was observed (Baxter et al., 1972; Kalimi et al., 1973; Litwack et al., 1973). It is not surprizing then that the binding of glucocorticoid-receptor complexes to isolated nuclei was developed as one of the first cell-free assays for activation for a number of glucocorticoid target tissues including, among others, thymus (Munck et al., 1972), lymphoma cells (Rosenau et al., 1972) and embryonic chick retina (Sarkar and Moscona, 1974), and is still used frequently. There are some potential drawbacks to the nuclear-binding assay, however. The tissue specificity of the binding of activated complexes to nuclei in cell-free systems is not high (Kalimi et al., 1973; Lippman and Thompson, 1974; Feldman et al., 1975; Romanova et al., 1983) and apparently the nuclear binding sites in cell-free systems are non-saturable (Simons et al., 1976; Milgrom and

Atger, 1975), suggesting the possibility that the nuclear sites to which glucocorticoid-receptor complexes are bound in cell-free systems may differ from those to which they are bound in intact cells which may be saturable (Higgins et al., 1973). This and the fact that the activated complex has an increased affinity for a number of different nuclear components including chromatin (Sakaue and Thompson, 1977; Simons et al., 1976), nucleosomes (Climent et al., 1977), DNA (Baxter et al., 1972; Rousseau et al., 1975; Sluyser, 1983), RNA (Tymoczko and Phillips, 1983), the nuclear matrix (Buttyan et al., 1983; Kirsch et al., 1986) and the nuclear membrane (Smith and von Holt, 1981; Kaufman and Shaper, 1984), has prevented a precise analysis of the physicochemical changes associated with activation based on the nuclear binding assay alone. In addition, the question of what exactly comprises the specific nuclear acceptor sites has not been resolved satisfactorily. Finally, much more time and care are required to prepare clean isolated nuclei than are required for various other activation assay systems.

Another, more defined system for quantifying and characterizing activation involves the binding of activated glucocorticoid-receptor complexes to DNA-cellulose (Alberts and Herrick, 1971; Kalimi et al., 1975; Luttge and Densmore, 1984). Advantages over nuclear binding assays include the ease of preparation (DNA-cellulose is available commercially) and the high degree of replicability obtained. An interesting observation was made by LeFevre et al. (1979) who reported that when the same hepatic cytosol containing heat-activated [3H]triamcinolone acetonide-receptor complexes was titrated by high concentrations of nuclei or DNA-cellulose, the former bound 75% of the complexes while the latter bound only 40%. These workers suggested that

this decreased binding was due on the one hand to a lower initial interaction between DNA-cellulose and activated complexes than between nuclei and these complexes and on the other hand to increased losses during washing when DNA-cellulose was used. However, these reasons can be discounted partially based on the results of Luttge and Densmore (1984) and Luttge et al. (1984 a,b,d) who used much longer incubation times to allow for increased interaction between DNA-cellulose and activated complexes, yet still observed a maximum binding of only 30-40%. In addition, these workers found losses during washes to be insignificant when washing pellets as many as 5 times. More likely explanations for the roughly two-fold difference between nucleus and DNA-cellulose binding include the possibility that free steroids may be binding directly to nuclei (Kaufman and Shaper, 1984) or that two forms of activated glucocorticoid-receptor complexes exist, both of which can bind to nuclei with relatively high affinity, but only one of which can bind to DNA-cellulose. Hirose and co-workers (1983) reported two such activated forms for rat mammary glucocorticoid-receptor complexes which they claim have slightly different sedimentation properties. These workers compared their findings to those of others who have studied extensively the progesterone receptor from avian oviduct. This progesterone receptor was shown to consist of two subunits, A and B, and that as nuclear acceptor sites, A and B subunits recognize DNA and chromosomal proteins, respectively. If such a situation does exist for glucocorticoid receptors, the separation and subsequent detailed characterization of each of the two or more forms of the activated glucocorticoid-receptor complexes would surely help to clarify a number of nuclear-receptor interactions and would probably provide much greater

resolution in the physicochemical characteristics determined for the glucocorticoid receptor. Further evidence for existence of multiple populations of activated glucocorticoid receptors was presented by Munck and Foley (1980) who showed that the "activated" peak eluted from a DEAE-cellulose column could be separated into two populations based on their binding to DNA-cellulose. Cidlowski and Munck (1980) used differential salt extraction of nuclei from cells incubated with glucocorticoids under various conditions to illustrate "that glucocorticoids form physiologically distinct classes of nuclear acceptor-receptor complexes."

Ion-exchange chromatography has proved to be a particularly useful tool for simultaneously separating activated from unactivated glucocorticoid-receptor complexes and providing information about surface charge changes during activation. A number of studies have employed the popular anion-exchangers diethylaminoethyl cellulose (DEAE-cellulose) and DEAE-Sephadex. Investigators in two labs reported simultaneously the successful separation of the unactivated and activated forms by rapid ion-exchange chromatography. Parchman and Litwack (1977) reported that the more acidic unactivated rat liver glucocorticoid-receptor complexes, which do not bind to carboxymethyl-Sephadex (CM-Sephadex, a cationic exchanger) or DNA-cellulose, are eluted from minicolumns of DEAE-Sephadex in phosphate buffer containing 0.4 M KCl. The more basic activated glucocorticoid-receptor complexes that bind to both CM-Sephadex and DNA-cellulose are eluted from DEAE-Sephadex at a lower salt concentration (0.2 M KCl). The fact that this form of the receptor bound to both DEAE- and CM-Sephadex indicates the presence of both positively and negatively charged regions on its

surface. Since it was more easily eluted from CM-Sephadex, it is probably an acidic protein with a localized basic region. Sakaue and Thompson (1977) resolved activated and unactivated glucocorticoid-receptor complexes by chromatography on DEAE-cellulose with potassium phosphate as the eluting salt. With this anion-exchange resin the peak of radioactivity corresponding to the unactivated complexes, which do not bind to either DNA or chromatin, was eluted by 0.24 M potassium phosphate, whereas the peak corresponding to the activated complexes, which bind to nuclei, chromatin and DNA, is eluted by 0.06 M potassium phosphate. These investigators reported that virtually identical chromatographic results were obtained with extracts from rat HTC and human LA9 cells, rat thymus and rat brain, indicating that the procedure can apparently be used to study glucocorticoid-receptor complexes in a variety of responsive tissues. Recently, DEAE-cellulose chromatography has been used as a step in the purification of unactivated glucocorticoid receptors (Manz et al., 1983), and DEAE-Sepharose has been used in the purification of activated receptors (Wrange et al., 1984).

Another chromatographic procedure with potential for studying the process of glucocorticoid receptor activation is dye-ligand affinity chromatography. Perhaps the most commonly used dye-ligand for protein purification has been Cibacron Blue F3GA, originally a textile dye and now refered to as a "universal pseudoaffinity ligand" (for review see Subramanian, 1984). Cibacron Blue was initially thought to possess a discriminating ability to bind to selected proteins containing a specific structure called a "dinucleotide fold", often associated with the ability of a protein to bind nucleotides. Although the dye eventually proved to be less specific for dinucleotide fold-containing

proteins than originally thought, it was shown to inhibit the binding of activated mouse uterine estradiol receptors to oligodeoxynucleotide cellulose (Kumar et al., 1979), and the activated and unactivated forms of the glucocorticoid receptor have been shown to bind differentially to Cibacron Blue affinity columns. Mouse brain glucocorticoid receptors have also been observed to bind to Cibacron Blue (Blue A)-agarose columns with apparent high affinity (Luttge and Densmore, unpublished) and affinity immobilization on Cibacron Blue-Sepharose 6B has been used most recently as a microassay for the determination of various binding parameters of estrogen and androgen receptors (Iqbal et al., 1985). Until the precise nature of the interaction between the receptor and Cibacron Blue F3GA is known, however, results from such studies can be of only limited value in the interpretation of the qualitative aspects of steroid receptor activation.

Although a change in the sedimentation coefficient (typically 9-10 S to approximately 3-4 S) of the glucocorticoid-receptor complex is considered by many to be associated with the process of activation, published reports of sedimentation data have often been confusing and inconsistent. Excluding known proteolytic degradation products, sedimentation coefficient values of 3.2-3.5S (Wrange et al., 1979; Raaka and Samuels, 1983; O'Brien and Cidlowski, 1981), 3.8-4S (Luttge et al., 1984a,b,d; Eisen and Glinsman, 1978; Beato and Feigelson, 1972; Carlstedt-Duke et al., 1977; Norris and Kohler, 1983; Middlebrooks and Aronow, 1977), 4-6S (Sherman, 1984; Holbrook et al., 1983a; Weatherill and Bell, 1985; Alexis et al., 1983; Vedeckis, 1983; Raaka and Samuels, 1983), 6-8S (Raaka and Samuels, 1983; Beato and Feigelson, 1972; Baxter and Tomkins, 1971; Kalimi et al., 1975), 8-9S (Failla et al., 1975;

Vedeckis, 1983; Middlebrooks and Aronow, 1977) and 9-10S (Alexis et al., 1983; Sherman, 1984; Holbrook et al., 1983a; Raaka and Samuels, 1983; Norris and Kohler, 1983; Grandics et al., 1984b; Luttge et al., 1984a,b,d) have been reported. While tissue and species differences can not be ruled out as being at least partially responsible for these inconsistencies, widely variable experimental conditions are certainly a factor. Variation in the ionic strength, which is well known to effect the hydrodynamic properties of proteins (Sherman, 1984), is common from one study to the next. Perhaps even more important is the fact that most earlier studies ran gradients that provided no protection for the receptor against either activation or inactivation. Therefore receptor complexes that had initially been identified as unactivated prior to going on a gradient might have undergone activation during the long centrifugation (typically 16-24 hours). Since the rate of activation would increase as the salt concentration of the gradient increased, it is easy to see why there were so many difficulties associated with the interpretation of these studies. One method of dramatically shortening the long centrifugation runs, but which has seen very limited use, involves the use of a vertical tube rotor. Run times on vertical rotors (typically less than 2 hours) can be reduced to between one-fifth and one- thirteenth the time required for swinging bucket rotors. One possible reason why vertical tube rotor sucrose gradient ultracentrifugation has been utilized much less frequently since its first reported use in the study of steroid hormone receptors (Hofman et al., 1978) is that the technique has been claimed to result in poor resolution of various receptor forms (Traish et al., 1981). However, recent reports have described conditions which yield very good

resolution of the multiple forms of the mouse glucocorticoid receptor (with peaks at 3.8 S, 5.2 S and 9.1 S) which occur before, during and after the activation process (Eastman-Reks et al., 1984; Reker et al., 1985). The technique has also been reported to produce good results with progestin receptors (Schneider et al., 1984).

In agreement with the decrease in sedimentation coefficient of the receptor complex associated with activation, are reports of decreases in both the apparent molecular mass and Stoke's radius during activation. The Stoke's radius of the unactivated form from mouse brain decreased from 7.7 to 5.8 nm during activation, while the apparent molecular mass decreased from 297,000 to 92,000 daltons as determined by Sephacryl S-300 gel exclusion chromatography (Luttge et al., 1984d). This agrees closely with the recent findings of Gehring and Arndt (1985), who reported molecular weights of 325,000 and 94,000 daltons for the unactivated and activated forms of the S49.1 lymphoma cell glucocorticoid receptor as also determined by Sephacryl S-300 chromatography.

Most recently, gel exclusion high performance liquid chromatography (HPLC) has been employed to characterize steroid receptors and the physicochemical changes they undergo during activation. The advantages of HPLC over conventional (open-column) gel exclusion chromatography include a decrease in the time required to separate the various forms of the receptor and an apparent increase in the resolution. Wiehle et al. (1984) used gel exclusion HPLC to separate estrogen, progestin and androgen receptors from several target tissues within 50 min on the basis of size and shape (Stoke's radius). These workers reported that the system provided for the detection of heterogeneity of receptor forms

in a manner superior to that observed with sucrose density gradient centrifugation. Barbey et al. (1983) achieved similar results with estrogen, progesterone and glucocorticoid receptors from a large number of breast cancer cytosols, detecting five forms each for progesterone and glucocorticoid receptors, and six forms for estrogen receptors. Again, these researchers found the HPLC results favourable to those obtained with either conventional gel exclusion chromatography or density gradient ultracentrifugation. However, recently it has been reported that certain forms of the glucocorticoid-receptor complex extracted from mouse AtT-20 cells appeared to be less stable during a 15-20 min HPLC run than during a 65 min vertical tube rotor sucrose density gradient ultracentrifugation run (LaPointe and Vedeckis, 1984; LaPointe et al., 1986). The instability of a 5.2 S intermediate form of the receptor during the short HPLC run was unaffected by the presence or absence of sodium molybdate. Hutchens et al. (1984) reported that gel exclusion HPLC of estrogen binding proteins from human uterine cytosol demonstrated a predominant 8-8.5 nm species, whereas conventional size exclusion chromatography revealed two to three distinct regions of estrogen binding proteins with Stokes radii of about 8.5, 6 and 3 nm (major species). Unlike the previously cited reports concerning HPLC, however, this study did not include molybdate in the elution buffers, and therefore an increased lability (or susceptibility to activation) of the larger forms of the receptor would be much more apparent during the very long open-column chromatography runs than during the very short HPLC runs.

Other means by which activation-induced changes in steroid receptor structure have been studied include polyacrylamide gel electrophoresis

and isoelectric focusing. Like vertical rotor sedimentation analysis and HPLC, these techniques have been used rather infrequently for the characterization of steroid receptors. Ben-Or and Chrambach (1981) were able to resolve three molecular species of glucocorticoid receptors from chick neural retina by gel electrophoresis (see also Ben-Or, 1983). These same workers later used isoelectric focusing techniques along with molybdate containing buffers to determine the apparent isoelectric points of three major forms of the receptor (ranging from 5.4 to 7.6) and the relationship between each of these forms and the process of receptor activation (Ben-Or and Chrambach, 1984). Cidlowski and Richon (1984) more recently combined these two techniques for a two-dimensional gel analysis of affinity labeled (dexamethasone mesylate) human glucocorticoid receptors by first subjecting the receptors to isoelectric focusing and then to SDS-polyacrylamide gel electrophoresis. Their data suggested that the human glucocorticoid receptor consists of a family of at least five proteins with molecular masses of approximately 88,000 which have discrete isoelectric points ranging from 6.5 to 7.5, possibly representing post transcriptional modification of a single protein. However, when a nonaffinity label (such as dexamethasone) was used, such microheterogeneity was not seen and only a single isoelectric peak of about 6.2 was detected for hippocampal or renal glucocorticoid receptors from rat (Wrange and Yu, 1983). However, this same lab (Wrange et al., 1984) has more recently used sodium dodecyl sulfate-gel electrophoresis to characterized purified activated glucocorticoid receptor from rat liver cytosol. They reported finding 94,000, 79,000, and 72,000 molecular mass species of the activated receptor. Another investigator has used polyacrylamide gel-electrophoresis to measure

various kinetic binding parameters of glucocorticoid receptors from hepatoma cells (Lenger, 1983).

Only recently, however, has there been any attempt to activate highly purified unactivated glucocorticoid receptors. The investigation of activation under purified conditions should provide important new clues regarding the potential role of endogenous factors, enzymes, etc. in the activation process(es). Although the reported results vary somewhat, perhaps dependent upon the degree of purification, one finding seems to be relatively consistent. It has been shown in at least three different studies that activation of purified receptors is not followed by the same increase in binding to DNA as is the case with unpurified receptor preparations (Grandics et al., 1984b; Webb et al., 1985; Schmidt et al., 1985). It has been shown by each of these studies that unpurified cytosol will dramatically increase the binding of prepurified activated glucocorticoid receptors when added to the preparation, although the nature of the cytosolic factor(s) responsible for increasing DNA binding are currently unknown.

Materials and Methods

Chemicals, Steroids and Isotopes

[6,7-3H]Triamcinolone acetonide, or 9a-fluoro-11b,16a,17a,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide, ([3H]TA, specific activity = 37 Ci/mmol) and [6,7-3H]dexamethasone, or 9a-fluoro-16a-methylprednisolone ([3H]DEX, specific activity = 44.1-48.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Sterogel A affinity resin (deoxycorticosterone agarose) was purchased from Sterogene Biochemicals. Sephadex G-25 (fine) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Dithiothreitol (DTT), and

4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) was courtesy of Research Organics (Cleveland, OH). Sodium molybdate (Na2Mo04), calf thymus DNA-cellulose, glycerol, sucrose, PPO (2,5-diphenyloxazole) and dimethyl POPOP (1,4-bis[2(4-methyl-5-phenyloxazoyl)]benzene) and pentyl agarose were all purchased from Sigma Chemical Co. (St. Louis, MO). Scinti Verse II was purchased from Fisher, Inc. (Fair Lawn, NJ). All other chemicals and solvents were reagent grade.

Animals

All studies used female CD-1 mice (Charles River Laboratories, Wilmington, MA) that were subjected to combined ovariectomy and adrenalectomy approximately 1 week prior to each experiment in order to remove known sources of endogenous steroids. Both operations were performed bilaterally via a lateral, subcostal approach under barbiturate anesthesia, and mice were given 0.9% NaCl (w:v) in place of drinking water. On the day of the experiment mice were anesthetized with ether and perfused slowly through the heart with ice-cold HEPES-buffered saline (20-30 ml, isotonic, pH 7.6).

Cytosol Preparation and Steroid Binding

Brains were removed from the perfused animals and homogenized (2x10 strokes at 1000 rpm) in 4 volumes of ice cold Buffer A (20 mM HEPES, 2 mM DTT and 20 mM Na2Mo04, pH 7.6 at 0 C) in a glass homogenizer with a Teflon pestle milled to a clearance between the pestle and homogenization tube of 0.125 mm on the radius (to minimize rupture of the brain cell nuclei (McEwen and Zigmond, 1972). The crude homogenate was centrifuged at 100,000 g for 20 min and the supernatant recentrifuged at 100,000 g for an additional 60 min to yield cytosol. During these centrifuge runs, and during all other procedures, unless

otherwise indicated, careful attention was paid to maintaining the cytosol at 0-2 C. Final protein concentrations were typically in the 6-8 mg/ml range. For experiments involving unpurified occupied (activated or unactivated) receptors, cytosol samples were incubated with 20 nM [3H]TA or [3H]DEX for 24 to 40 hours at 0 C with or without a 200-fold excess of unlabeled steroid.

Affinity Chromatography

Unlabeled cytosol was applied to small columns containing 1-2 ml of Sterogel affinity resin and incubated at 0 C for 2 to 8 hours prior to elution of the column with a minimum of 10 volumes of HEPES buffer containing 2 mM DTT and 50 mM molybdate. Direct steroid exchange was then carried out by incubating the washed resin with either [3H]DEX or [3H]TA (concentrations varied from 100 to 500 nM) for periods varying from 20 to 72 hr. Radiolabeled receptors were then eluted from the column using the previously described buffer and then subjected to Sephadex G-25 chromatography to remove free steroid. Samples were then either used for subsequent experimental analysis, counted to determine specific binding or assayed for protein content. Indirect steroid exchange involved first incubating the washed resin with 5 uM [1H]DEX for 4 hr, followed by elution from the affinity column and separation of free [1H]DEX on a Sephadex G-25 column prior to a second steroid exchange reaction involving incubation of the [1H]DEX-labeled receptors with 50 nM [3H]DEX or [3H]TA for periods varying from 20 to 72 hr.

Activation and Removal of Unbound Steroid

[3H]TA-labeled purified receptor preparations or [3H]TA-labeled cytosol samples (for experiments involving unpurified receptor preparations) to be activated were first run on Sephadex G-25 columns

(0.6 x 14 mm) equilibrated with buffer containing 20 mM HEPES and 2 mM DTT only. This column run resulted in the removal of both free steroid and molybdate, allowing for uninhibited activation (Luttge et al., 1984a,b,d; Luttge and Densmore, 1984). The bound fraction was then incubated at 22 C for variable periods of time to determine the minimum period of time required to achieve total activation. The degree of activation was determined by DNA-cellulose binding assay. For the purpose of hydrophobic interaction chromatography of activated receptors, the maximally activated receptor preparation was again run on a second Sephadex G-25 column equilibrated with the appropriate buffer (the same buffer used to equilibrate and elute the subsequent alkyl agarose columns). For unactivated receptors, the cytosol was treated in an identical fashion except that the first Sephadex G-25 column was equilibrated and eluted with HEPES buffer containing 20mM molybdate and 2 mM DTT and the bound fraction was subjected to a 0 C (rather than a 22 C) incubation prior to running on the second Sephadex G-25 column (identical to the second column run for the activated preparation).

DNA-cellulose Binding Assay

Calf thymus DNA-cellulose, prepared originally by the method of Alberts and Herrick (1971), was added to HEPES buffer containing 2 mM DTT to yield a final concentration of 10 mg/ml (4.1 mg DNA/g DNA-cellulose). In a typical assay (run as duplicates) a 100 ul aliquot of [3H]TA-labeled cytosol or purified receptor preparation was added to 300 ul of the DNA-cellulose slurry. The mixture was then vortexed gently and incubated at 0 C for 60 min. Assay tubes were oscillated at 150 rpm throughout the incubation and vortexed gently every 10 min. The DNA-cellulose was collected by centrifugation (2000 x g for 5 min), the

supernatant discarded and the DNA-cellulose pellet washed 3 times with 1 ml of HEPES buffer plus 2 mM DTT. The entire pellet was resuspended in deionized water and transferred to scintillation vials for determination of bound radioactivity. DNA-cellulose was prepared in buffer not containing molybdate in order to reduce the concentration of molybdate during the binding assay, since high concentrations of molybdate were found to reduce the efficacy of receptor binding to DNA-cellulose (Luttge et al., 1984b).

Hydrophobic Interaction Chromatography

[3H]TA-labeled purified receptor preparations were run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer containing 600 mM KCl and 50 mM molybdate at pH 7.6. Of the bound fraction collected from these columns, 0.5 ml was then run on a 7 ml pentyl agarose column equilibrated and eluted with HEPES buffer containing 600 mM KCl and 50 mM molybdate at pH 7.6. Fractions (0.5 ml) were collected and assayed for specific binding.

Sucrose Density Gradient Sedimentation

Purified receptor samples (400 ul) were layered onto linear 5-20% sucrose density gradients (4.6 ml; prepared with HEPES buffer containing 2 mM DTT and 20 mM molybdate) and centrifuged at 0 C for 1.5-2 hr at 370,000 g (average) in a Sorvall TV-865 vertical tube rotor. The cellulose nitrate tubes were punctured and 26-28 fractions (180 ul) collected and assayed for radioactivity by liquid scintillation spectrometry. Sedimentation coefficients (S20,w) were calculated from the linear regression of S20,w vs sedimentation distance (Martin and Ames, 1961) for the following standard proteins run in parrallel tubes: chicken ovalbumin (OVALB, 3.6 S), bovine serum albumin (BSA, 4.3 S),

bovine gamma globulin (IgG, 7.4 S) and catalase (CAT, 11.3 S). The standard proteins were [14C]methylated (for detection) to low specific activity with [14C]formaldehyde by the method of Rice and Means (1971).

Results

Initial attempts to purify the glucocorticoid receptor from brain using deoxycorticosterone agarose followed very closely the "batch" procedure originally described by Grandics et al. (1984b) for the purification of glucocorticoid receptors from liver except that HEPES buffer was used instead of potassium phosphate for cytosol preparation and for ecomomic reasons, a 1 uM concentration instead of a 2 uM concentration of [3H]TA was used for the steroid exchange reaction. For reasons that are not entirely clear, this procedure resulted in relatively low yields of purified receptors in the range of 5%, much lower than the 50-70% recoveries reported by Grandics et al. (1984b). While the 50% lower concentration of [3H]steroid used in the exchange probably contributed to the lower yields, it is not likely to be entirely responsible for a 10- to 14-fold lower receptor recovery rate. Some modifications to the procedure involved running cytosol through a column of deoxycorticosterone agarose as opposed to using the batch approach. In addition, it was assumed that due to the excellent stability of the [3H]TA-receptor complex in cytosol under these particular buffer conditions and because of the relatively very slow dissociation of [3H]TA (the free ligand) as compared to deoxycorticosterone (the matrix-associated ligand), a much higher yield should be obtained for a given concentration of [3H]TA if a much longer exchange incubation is used.

The next series of experiments involved applying a volume of cytosol to the affinity resin column which was roughly equivalent to the void volume of the column, thereby exposing all of the gel to receptor-containing cytosol. The columns were allowed to incubate for approximately 8 hr at 0 C prior to washing the affinity resin with at least 10 times the bed volume of the column. The column was washed with a buffer identical to that used in the preparation of cytosol containing both molybdate (to stabilize and prevent activation) and DTT (to prevent oxidation of any dissociated receptors which would preclude reassociation to either the affinity resin or the tritiated exchange ligand). Aliquots of each were checked for glucocorticoid binding activity by incubating with 20 nM [3H]TA +/- 4 uM [1H]TA for an additional 24 hr. No binding activity whatsoever was detected in any of these column washes indicating that the concentration of glucocorticoid receptors in cytosol as prepared for these experiments was far from saturating the affinity resin and that the incubation time of 8 hr at 0 C was more than sufficient to achieve virtually 100% binding of all receptors. After extensive washing of the column to remove any non-binding or low affinity-binding components, the steroid affinity gel was incubated with a lower concentration of [3H]TA (100 nM) than was used before, but the steroid exchange incubation period was extended considerably from 16 hr to 48 hr. The recovery of glucocorticoid receptors from brain cytosol was up from approximately 5% to 15%, a 3-fold increase over the previous procedure. When liver cytosol was used in these experiments, a similar yield of 15-20% was obtained but much more binding was obtained since the concentration of glucocorticoid receptors per gram wet weight of tissue is several times greater for liver than brain.

It was observed in subsequent studies using the purified receptor preparation obtained by the previously described procedure that these [3H]TA-labeled receptors appeared to be far more labile than unpurified [3H]TA-labeled receptors in cytosol. The dissociation rate for [3H]TA is extremely slow in cytosol with a half time of days at 0 C (Gray, 1982). Likewise, the rate of receptor degradation leading to an irreversible loss in binding is very slow at low temperatures, especially in the presence of molybdate and is generally not a factor in most experiments. However, for reasons that are not yet clear, affinity resin-purified [3H]TA-labeled glucocorticoid receptors undergo an approximately 50-60% loss at 0 C, even when excess free [3H]TA is present at concentrations greater than 100 nM, indicating that the loss probably represents an irreversible loss of binding capacity independent of steroid dissociation. Similar losses were seen when [3H]DEX was used as the exchange steroid. This finding had important implications for both the purification of glucocorticoid receptors and the subsequent studies regarding activation of the glucocorticoid receptor.

Due to the extended time and expense required for receptor purification and the unexpected lability under supposedly ideal conditions, there was an obvious need for reducing the losses in receptor binding until subsequent experimental manipulations could be carried out. One possibility was to freeze the receptor preparation immediately after purification, store at a very low temperature and thaw immediately prior to experimental use. High enough concentrations of purified bound receptor would have to be used in the subsequent experimental procedures to allow for detection of chromatographic peaks,

DNA-cellulose binding, etc. after substantial losses during some of the procedures. A series of experiments were therefore carried out to determine the lability of glucocorticoid binding in non-steroid-labeled cytosol, steroid-labeled cytosol and an affinity resin-purified preparation of unactivated receptors during freezing/thawing and during long-term storage in a deep-frozen state (-75 to -85 C). In addition, the effect of glycerol (0, 10 and 30% w:v) on glucocorticoid receptor lability under these conditions was simultaneously investigated. The initial part of the study involved unpurified receptor preparations (standard cytosol preparations) to provide, first, a comparison for purified preparations and second, potentially practical information for preparatory purposes when dealing with unpurified preparations. Cytosol prepared in the usual manner was split into two equal fractions for the two parts of this experiment, that being radiolabeled with [3H]TA prior to freezing and thawing (for purposes of studying the occupied form of the receptor), and that being radiolabeled with [3H]TA after freezing and thawing (for purposes of studying the unoccupied receptor). Appropriate volumes of glycerol and/or deionized water were added to subfractions of each of these groups to achieve the desired concentrations of glycerol while maintaining the concentration of cytosolic protein as well as other endogenous and exogenous compounds equal in all of these subfractions. The effects of freezing and thawing on the unoccupied receptor were determined by placing the unlabeled cytosolic samples in a -85 C freezer and subsequently thawing at various time intervals prior to incubation with 20 nM [3H]TA +/- 200-fold concentration of unlabeled TA (for determination of nonspecific binding). Since the melting point for all the groups, including the

nonglycerol group, was below 0 C, thawing was accomplished by simply placing the frozen sample tubes in an ice water bath at 0 C. Thawing took somewhat longer for the nonglycerol group, but in every case took no more than 15 min. The thawed samples were incubated with the indicated steroids for 24 hours prior to bound/free steroid separations performed on Sephadex G-25 columns. The effects of freezing and thawing on the occupied receptor were studied by first radiolabeling the cytosol (again with 20 nM [3H]TA +/- 4 uM [1H]TA) and then subjecting the labeled cytosol to freezing at -85 C followed by thawing after various time intervals. Bound/free separations were performed immeadiately after the freeze/thaw step. Later, purified [3H]TA-labeled receptor preparations were treated identically to the prelabeled cytosolic fraction. It is clear from the results (Table 4-1) that the process of freezing and thawing led to only a slight loss (10-15%) in cytosolic binding which was almost completely preventable by the addition of 10% glycerol. Increasing the glycerol concentration to 30% further decreased the freeze/thaw-induced binding loss to virtually zero. Interestingly there was very little difference between the stability of the bound versus the unbound receptors in cytosol during freezing and thawing. Perhaps even more important was the fact that virtually no further losses in binding capacity were encountered once the cytosol was frozen for periods of up to 5 to 11 days. Purified [3H]TA-labeled receptors, on the other hand, appeared more susceptible to freeze/thaw-induced losses, even in the presence of glycerol, though glycerol did decrease the degree of loss slightly. However, despite the more dramatic loss of binding capacity associated with the freezing and thawing of purified preparations, the degree of loss was nevertheless

acceptable when compared to the rapid rate of loss in an unfrozen state and further losses during long term storage at -85 C were almost negligible.

The discovery of the relative instability of the bound purified glucocorticoid receptor led to yet another change in the affinity purification procedure in an attempt to increase the yield of purified receptor. This involved reducing the steroid exchange incubation period to an intermediate level from 48 to 30 hr and again increasing the concentration of [3H]TA from 100 nM to 500 nM. These changes were based on the assumption that the increase in receptor lability occurs immediately after washing the affinity column free of non-binding components and that extended steroid incubations under these conditions would result in receptor degradation that would eventually outpace the rate of exchange between deoxycorticosterone and [3H]TA binding to the glucocorticoid receptor. The rate of exchange could be increased during a shorter incubation period by increasing the concentration of free [3H]TA relative to the concentration of resin-associated deoxycorticosterone. Although increasing the temperature would increase the rate of steroid exchange, it would simultaneously increase the rate of receptor degradation (the temperature dependence of purified receptor lability will be discussed later in this section). An additional step to enhance purified receptor stability and thereby increase recoveries during purification was to increase the concentration of molybdate from 20 to 50 mM in the buffers used in washing affinity columns and present during steroid exchange reactions and many subsequent steps. Although 20 mM molybdate is generally sufficient to almost completely inhibit both glucocorticoid receptor degradation that leads to a loss in binding

and activation of the steroid receptor complex to the nuclear binding form (Dahmer, 1983; Luttge et al., 1984a-d), a higher concentration of molybdate was found to further decrease the lability of the purified receptor, though only slightly (data not shown). The net result of these changes in the purified procedure was that a 20-45% recovery was now obtainable using both brain and liver. These recoveries, though still less than those reported by some labs (Grandics et al., 1984b) were finally in a range where ample quantities of purified receptor could be obtained for at least some physicochemical studies.

Since binding of the cytosolic glucocorticoid receptor to the affinity resin should occur very rapidly, because of a very high concentration of deoxycorticosterone attached to the resin, it was thought that even higher yields of purified receptor could be obtained even more economically by performing a two-step steroid exchange reaction. This involved an initial steroid exchange reaction, after washing the affinity resin free of non-binding components, with a very high concentration of [1H]DEX (4 uM) for approximately 8-12 hr. The [1H]DEX-labeled receptors, which after the incubation period should have represented a very large proportion of the total population of glucocorticoid receptors, were then eluted free of the affinity resin column prior to being subjected to Sephadex G-25 chromatography in order to remove free [1H]DEX from the bound purified receptors. The bound fraction was then incubated with 40 nM [3H]TA at 0 C for the second steroid exchange reaction. Aliquots of this preparation were periodically assayed for [3H]TA binding by again running bound/free steroid separations on Sephadex G-25 columns. Using [1H]DEX for the first exchange ligand served two purposes. First, DEX is roughly equal

to TA in its specificity for the type II glucocorticoid receptor (it should be noted that progesterone and type I adrenocorticoid (mineralocorticoid) receptors have some affinity for deoxycorticosterone). Second is the fact that DEX dissociates from the receptor much faster than TA (Gray, 1982). Since the [3H]TA had only to compete with the very low, almost negligible, levels of free [1H]DEX that had dissociated from glucocorticoid receptors in the preparation, much lower concentrations (40 nM versus 500 nM) could be used for this exchange and, theoretically, result in a high yield of [3H]TA-labeled receptor. Unfortunately, this two-step steroid exchange procedure did not result in any greater recovery than was obtained with a single-step steroid exchange, and, in fact, the maximal recoveries (12-20%) were typically about half of what had been achieved with the single-step process. One advantage of the procedure, however, is the lower cost per quantity of receptor recovered.

Another two-step exchange procedure for purifying glucocorticoid receptors attempted involved an intermediate sulfhydryl down-regulation step in order to remove the bound receptors from the affinity column in an unoccupied state. The procedure offered the potential for yet another economical method of glucocorticoid receptor purification, while also allowing for the possibility of obtaining fairly pure glucocorticoid receptors in an unoccupied form, another goal of this research. This procedure involved first incubating brain or liver cytosol with the deoxycorticosterone agarose as previously described followed by extensive washing of the column to remove non-binding components. A change in the washing procedure from previously described experiments involved using a buffer not containing DTT during the final

3-4 washes. This non-DTT washing step was then followed by incubation of the affinity column with 10 mM DTNB. DTNB had previously been shown to reversibly down-regulate the binding capacity of unoccupied glucocorticoid receptors by apparently interacting with a binding site sulfhydryl group(s) while having no effect on prebound receptors (see Chapter III). It was assumed that the free DTNB would compete with the matrix-associated deoxycorticosterone for the steroid binding site of dissociated receptors, down-regulating them and preventing any further rebinding to the steroid affinity matrix. The down-regulated unoccupied receptors were then washed free of the affinity matrix and the resulting preparation was run on a Sephadex G-25 column in order to remove any free DTNB. The macromolecular fraction was then incubated with 20 nM [3H]TA in the presence of 10 mM DTT, which had previously been shown to up-regulate virtually 100% of unoccupied receptors down-regulated in cytosol by DTNB. For reasons that are not entirely clear, absolutely no [3H]TA binding was ever detected, even after several modifications of the procedure involving incubation times, DTNB concentrations, etc.

Another modification of the procedure which greatly increased the quantity of receptor purified per ml of affinity resin was based on the high concentration of deoxycorticosterone attached to the gel matrix. It was found that when the affinity column was continually "washed" with yet another application of the original cytosol, allowing for 2 to 4 hr of incubation at 0 C between each application of cytosol. Up to 10 volumes of cytosol were applied to the affinity resin before the wash procedure (using buffer only) was initiated. All washes eluting from the column were checked for glucocorticoid binding activity by incubating aliquots with 20 nM [3H]TA +/- 4 uM [1H]TA for 24 hr at 0 C.

No [3H]TA binding was ever detected, indicating that the affinity column had not been saturated with receptors even after 10 applications of cytosol. Another positive point regarding this procedure is that receptor lability is not a problem during the entire multiple cytosol application step since the receptors bound to the affinity matrix are continuously stabilized by the cytosolic factors later removed by washing with buffer only. After the steroid exchange reaction with [3H]TA was complete, total recoveries were still in the 20-45% range meaning that multiple applications of cytosol onto the steroid affinity resin resulted in up to 10 times the total amount of receptor normally obtainable per ml of resin with a single application of cytosol. It is likely that even more cytosol could be applied to the gel, resulting in even greater quantities of purified receptor per ml of affinity resin per experiment, but this was not further investigated in the present study.

Activation of the steroid affinity resin-purified glucocorticoid receptor was carried out by using procedures nearly identical to those described previously by this lab for the activation of cytosolic [3H]TA-labeled glucocorticoid receptors (Luttge et al., 1984a-d; see also Chapter II). Previously frozen samples of purified receptor were thawed by incubation in a 0 C water bath, then run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer containing 2 mM DTT and either containing or not containing 50 mM molybdate. The bound fraction collected from the Sephadex G-25 column equilibrated with non-molybdate-containing buffer was subjected to a 24 min incubation at 22 C after which the sample was again cooled quickly to 0 C and sufficient molybdate was added to achieve a final concentration of 50

mM. Both activated and unactivated preparations were then again run on Sephadex G-25 columns equilibrated and eluted with a buffer of variable composition depending upon the particular experiment that followed. This second column run acted to reequilibrate both activated and unactivated preparations in the same buffer and also acted to remove any free steroid occurring as the result of receptor degradation resulting in steroid dissociation. One finding that was immediately apparent was that heat-induced activation led to a significant loss (approximately 50-75%) in receptor binding, far more than generally had been observed during the activation of cytosolic glucocorticoid receptors (Luttge et al., 1984a-d; Luttge and Densmore, 1984). This finding resulted in the use of higher concentrations of purified receptor to allow for expected losses in binding during heat activation and subsequent activation assay procedures.

Initial attempts to activate the purified receptor investigated changes in binding of the receptor to DNA-cellulose associated with activation. The first experiment looked at DNA-cellulose binding before and after the single activation incubation previously described (24 min at 22 C). Unpurified cytosolic receptor preparations, run simultaneously as controls, displayed the expected changes in DNA affinity upon activation (less than 1% of unactivated unpurified receptors bound to DNA-cellulose whereas 45-50% of the activated unpurified receptors bound to DNA-cellulose, a greater than 50-fold increase). Interestingly, the purified unoccupied receptor sometimes appeared to have a higher affinity for DNA-cellulose (2-10% binding) than did the cytosolic unoccupied receptor (<1% binding), but the purified activated receptor always displayed precisely the same affinity

for DNA-cellulose as did the purified unactivated receptors within a given experiment. This slight and variable binding of purified receptor to DNA-cellulose is not completely understood, but it was clear after several replications that no further increases in binding were associated with heat activation under the conditions tested.

The possibility that activation of purified receptors may require conditions different from those needed for activation of unpurified receptors was investigated in part. While the temperature of activation (22 C) was left constant, the duration of activation was varied to include periods that were both shorter (6 and 12 min) and longer (48 min) than the original incubation period of 24 min. In addition, purified receptor groups were included at each of those time points which were activated in the presence of 150 mM KCl to determine if ionic strength was a more important factor in the activation of purified receptors. The KCl, where added during activation, was removed from the samples during the run on the second Sephadex G-25 column which was always equilibrated and eluted with HEPES buffer containing 2 mM DTT and 20 mM molybdate. It should be noted that all of the samples were equilibrated to 20 mM molybdate instead of 50 mM molybdate, the concentration which had been used during the purification procedure, storage, etc., because this lab had previously shown higher concentrations of molybdate to inhibit the binding of activated glucocorticoid receptors to DNA-cellulose (Luttge et al., 1984b). The results of this limited study (Table 4-2) showed that neither duration of activation nor ionic strength were important factors in the activation of the purified glucocorticoid receptor as measured by DNA-cellulose binding. The increased heat lability of the purified

receptor made interpreting the DNA-binding data for the 48 min activation a problem since the total level of [3H]TA binding was reduced to the point that a small change in the already small percentage of receptor bound to DNA-cellulose was hard to determine accurately. Again, however, any major increases in binding would have been evident, but they were not. The effect of including 150 mM KCl during the 22 C activation incubation on DNA-cellulose binding seemed to also be negligible. The change in ionic strength also had virtually no effect on the temperature-dependent lability of the purified glucocorticoid receptor.

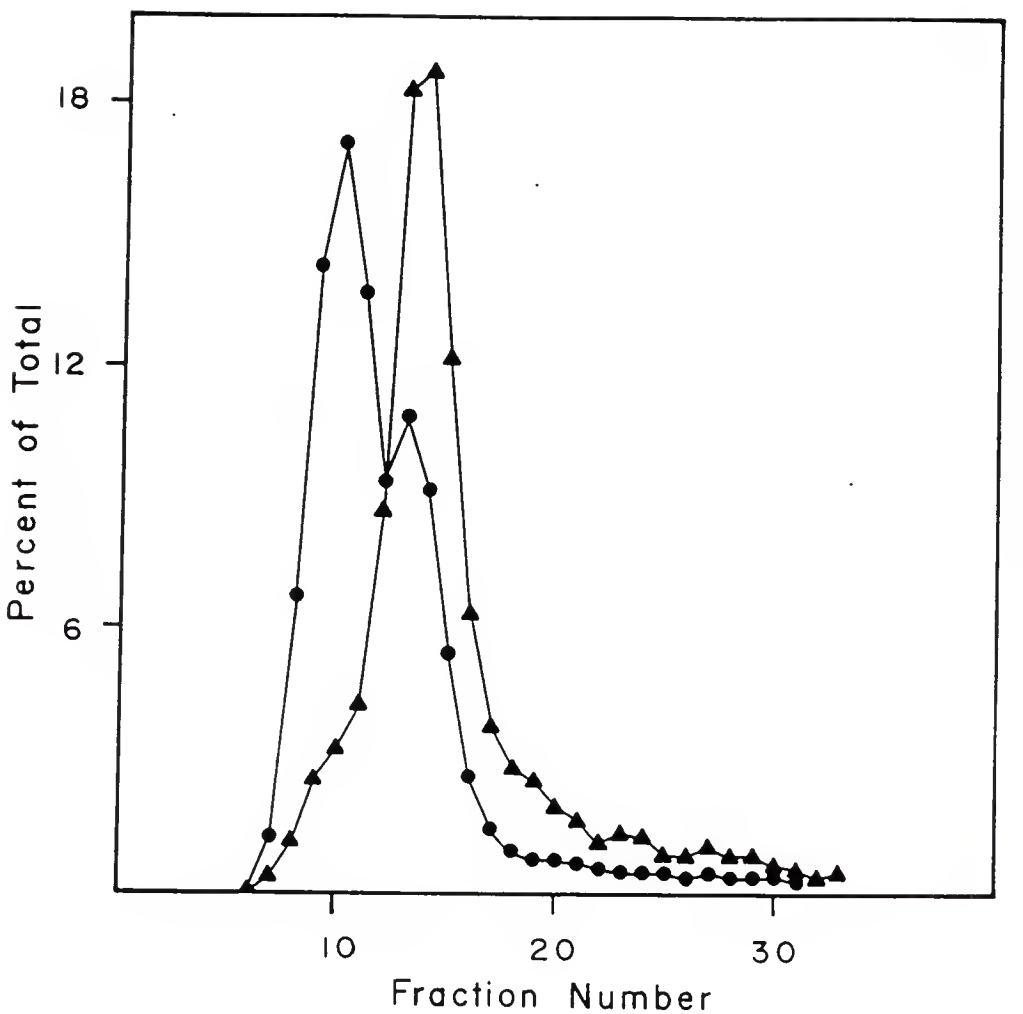
In order to determine if the inability of activated purified glucocorticoid receptors to bind to DNA represented a purification-induced modification in only the DNA binding properties of the receptor or an overall inability of the receptor to undergo the major physical changes generally associated with activation, other means of monitoring changes in the properties of the receptor associated with heat activation were employed. One of the analytical tools used to study this phenomenon (or lack thereof) was hydrophobic interaction chromatography. Measurements of surface hydrophobicity had previously been used to differentiate between activated and unactivated glucocorticoid in unpurified preparations of brain cytosol (see Chapter II). The current investigation of purified receptors used a 7 ml pentyl agarose column of the precise demensions previously used in the hydrophobic analysis of unpurified receptors. Again, the purified receptor preparation was activated in the absence of molybdate at 22 C for 24 min prior to being run on a Sephadex G-25 column equilibrated and eluted with HEPES buffer containing 600 mM KCl and 50 mM molybdate which

was also used to equilibrate and elute the pentyl agarose column. The profiles of both the purified activated and unactivated receptors (Figure 4-1) were strikingly different from the profiles obtained with their unpurified counterparts. Perhaps even more interesting is the fact that the purified activated and unactivated profiles differed significantly from one another. The purified unactivated receptor resulted in a profile containing one major and one minor peak. The major peak eluted first and at the identical position that the unpurified unactivated receptor eluted at under identical conditions (Figure 2-5). The minor peak eluted only 3 fractions later and represented a fraction of the total binding that varied slightly from one replication to the next but averaged approximately 25%. Both peaks were relatively sharp and well defined and their positions were very replicable. The profile of purified activated receptor was a single sharp peak eluting at the same position as the second minor peak of the purified unactivated receptor profile. While all of the peaks obtained with the purified preparations were sharper than those obtained with the unpurified preparations, the greatest contrast was between the purified and unpurified activated profiles. The unpurified activated peak is dramatically broader and elutes slower than its purified counterpart.

Discussion

This section of the dissertation sought primarily to investigate the process of receptor activation using a receptor preparation as free as possible of non-receptor cytosolic components that could interfere with, or contribute to, the conversion of the receptor from a nuclear non-binding to a nuclear binding form. The first step required for such an investigation was the purification of the unactivated form of the

Figure 4-1. Hydrophobic interaction chromatography on purified samples of unactivated and activated Type II glucocorticoid receptors. Brain cytosol prepared in HEPES buffer containing 50 mM molybdate and 2 mM DTT was applied to a deoxycorticosterone agarose affinity column and incubated for 4 hr at 0 C. The column was then washed free of nonbinding components using 10 washes of the same buffer and then incubated for 40 hr at 0 C with 250 nM [3H]TA to facilitate a steroid exchange. The purified receptors, labeled with [3H]TA, were then eluted with the original buffer and run on Sephadex G-25 columns equilibrated and eluted with HEPES buffer plus 600 mM KCl and 50 mM molybdate. Aliquots (0.5 ml) from the macromolecular fractions collected from these columns were then run on 7 ml pentyl agarose columns equilibrated and eluted (0.5 ml fractions) with the same HEPES, KCl, molybdate buffer. Binding is expressed as percent of the total counts (12,000 cpm) applied to the column. The profile shown here is representative of two independent replications.


18
12
6
Percent of Total
10
20
30
Fraction Number

glucocorticoid-receptor complex. It was also important that the process of purification did not in itself lead to activation of the receptor. Although several labs have recently undertaken the task of purifying the glucocorticoid receptor (see introduction of this chapter for details), numerous experimental factors, which can vary from one lab to another have been shown to dramatically affect receptor stability, receptor activation, steroid association and dissociation. The effects of these variable factors on receptor parameters may actually be magnified by the process of purification since many inhibitors of receptor degradation and activation are removed from the preparation. For this reason, the methodology for unactivated glucocorticoid receptor purification had to be perfected in order to work effectively under the experimental conditions previously and currently used by this lab for a number of extensive studies regarding glucocorticoid receptor regulation and activation. Although receptor recoveries of 45% may have been somewhat lower than those reported by Grandics et al. (1984b) and Govidan and Gonemeyer (1984) which ranged from 50 to 70%, they were sufficiently high enough to carry out the subsequent investigations of activation-induced changes in purified glucocorticoid receptor properties. This is partly true because of the relatively high concentrations of glucocorticoid receptors in the tissues used for purification (brain and liver). In addition, only relatively low levels of receptor were needed to perform many of the qualitative and quantitative determinations.

Unfortunately, purificaton of unoccupied receptors using deoxycorticosterone agarose affinity chromatography proved entirely unsuccessful. Although the reasons for this are not entirely known,

these negative findings may, nevertheless, provide additional clues as to the nature of the purified receptor. All indications from other work in this dissertation regarding sulfhydryl down-regulation of glucocorticoid receptor binding capacity (see Chapter III) were that DTNB treatment of either liganded or unliganded receptors led to no irreversible loss of binding capacity, beyond what was normally encountered in the absence of DTNB, when all other conditions were constant. It therefore came as a surprise that DTNB appeared to irreversibly inactivate the binding capacity of purified glucocorticoid receptors on the affinty column. Even if the high concentration of DTNB had only been a poor competitor for the lower concentration of affinity resin-linked deoxycorticosterone, one would have expected some recovery of receptor binding capacity since the reaction between the receptor and DTNB is irreversible until DTT is added after the prepartion is washed from the column. Because the total amount of receptor binding activity associated with the affinity column is so high and because nonspecific binding activity removed from the column as a result of the DTNB incubation is, for all practical purposes, zero, specific binding recoveries as low as 0.02 to 0.05% could have been easily and accurately detected. It can therefore be safely assumed that the inability to recover any binding activity by this method was not merely due to a concentration-related problem regarding the DTNB. It should also be noted that even if the method had been successful in purifying the unoccupied glucocorticoid receptor, the final preparation probably would not have been as pure or homogeneous as that obtained by the steroid-exchange purification of steroid-labeled receptors using the same steroid affinity resin. This is because the exchange with DEX or

TA is very specific for glucocorticoid type II receptors, whereas DTNB or other sulfhydryl reagents have been shown to reversibly inactivate the binding capacity of progesterone (Kalimi and Banerji, 1981) as well as glucocorticoid type I (Emadian and Luttge, unpublished) receptors, both of which also bind to deoxycorticosterone, but have very low affiinities for DEX or TA.

Since this work was done, a report has very recently appeared which described the partial purification of the ligand-free glucocorticoid receptor (Krajcsi and Aranyi, 1986). These workers used a cortexolone-substituted affinity matrix and eluted receptors from the column via an exchange with either free TA or cortexolone. Owing to the relatively fast dissociation of the glucocorticoid receptor-cortexolone complex, a partially purified free glucocorticoid receptor capable of steroid binding, was obtained by merely allowing for the rapid dissociation of the purified complex. It should be noted, however, that like deoxycorticosterone, cortexolone is not specific for only the glucocorticoid type II receptor (Krajcsi and Aranyi, 1986).

Using a dissociation approach to purification of unoccupied receptors probably would have resulted in some degree of success in the present study. However, the yields would have been lower than those for purified bound receptors because of binding losses encountered during the relatively long periods required for dissociation of DEX. Although purification recoveries as high as 20% were achieved with a two-step steroid exchange using DEX as the intermediate steroid, it should be remembered that receptors dissociated from DEX rapidly rebound TA. Since it was never determined in the present study what impact, if any, steroid binding has on the stability of the purified unoccupied

receptor, it would not be possible to predict what yields could have been achieved by such a procedure.

The successful long-term storage of both purified and unpurified (cytosol), steroid-bound and free glucocorticoid receptors at -75 to -85 C with virtually no losses in binding capacity provided an additional degree of convenience when running long, complex, multi-step experiments. The losses that did occur during the freezing and thawing were not considered a serioius drawback to cold storage and these losses could generally be eliminated, or nearly so, by the inclusion of the cryoprotective agent glycerol. The greater losses encountered during the freezing and thawing of purified receptor preparations, even in the presence of glycerol, were probably related to the absence of some endogenous component(s) acting in a cryoprotective manner. These missing factors may have some relation to the increased lability of the purified glucocorticoid receptor, even in the presence of molybdate. The losses are still acceptable, however, considering the importance of storage of a form that is so costly (in time and money) to prepare each time. McLusky et al. (1986) recently examined the effects of freezing and thawing on steroid receptor concentrations in the brain and pituitary of the rat. These workers found that freezing and thawing resulted in measurable losses of cytoplasmic androgen, progestin and glucocorticoid receptors, while estrogen receptors were relatively stable. They also found that in all cases except, interestingly, that of cytoplasmic glucocorticoid receptors, these losses could be prevented by freezing the tissue in 10% aqueous dimethylsulfoxide, another reagent with known cryoprotective properties. With regards to the effects of freezing on other properties of steroid receptors, Janes et al. (1982)

earlier compared the sucrose density gradient sedimentation profiles of uterine estrogen and progesterone receptors that were either frozen or lyophilized and found little difference between the two. Unfortunately, these workers did not compare their findings directly with those for fresh cytosolic preparations, but the implication was that freezing and thawing of either cytosol or whole tissue had little effect on subsequent receptor stability, activation, etc..

One of the primary reasons for purifying the glucocorticoid receptor was to allow for a more complete understanding of the process of activation of the receptor to its nuclear binding form. The relative importance of examining glucocorticoid receptor activation using a purified preparation is exemplified by a number of recent reports that appeared during the planning and progress of the present study (Grandics et al., 1984b; Wrange et al., 1984; Schmidt et al., 1985; Webb et al., 1985; Krajcsi and Aranyi, 1986; Schmidt et al., 1986). The finding of the present study that purified activated receptors have a dramatically reduced affinity for DNA is in general agreement with the findings of Grandics et al. (1984b), Schmidt et al. (1985 and 1986) and Webb et al. (1985). In contrast, Krajcsi and Aranyi (1986) were able to achieve an increase in DNA binding that was close to the levels attained when crude cytosol preparations were used. This is probably because of the fact that the cortexolone-substituted affinity procedure used by these workers was reported to result in a lower degree of purification (only 75 to 100-fold) than was achievable by the deoxycorticosterone affinity procedure used in the present study and by the other workers (several hundred to thousand-fold). It is likely that a very high degree of purification is required in order to remove whatever factor(s) are

involved in the transformation of the receptor to a form able to bind DNA.

Despite the lack of activation-induced increases in DNA binding, the finding in this study that heat activation did lead to changes in the purified receptor normally associated with activation of unpurified receptors, such as an increase in hydrophobicity, leads to the notion that activation may be a multi-step process and that some changes, such as hydrophobicity, are not dependent upon cytosolic factors removed by purification, whereas other changes, such as DNA binding, are. Such a possibility has also been suggested by Schmidt et al. (1985), who found that whereas purified glucocorticoid receptors underwent a temperature-dependent, molybdate-sensitive change in DEAE-cellulose binding, a heat-stable cytoplasmic macromolecule was required to subsequently achieve an increase in DNA binding. This second step involving cytoplasmic factor(s) was reportedly molybdate-insensitive and temperature-independent. More recently, a preliminary report by Schmidt et al. (1986) showed that bovine pancreatic ribonuclease (RNase) A and S-protein (an enzymatically inactive proteolytic fragment of RNase A which contains the RNA binding site) stimulated the activation, as evidenced by increased DNA-cellulose binding, of highly purified rat hepatic glucocorticoid receptors. These authors concluded that their findings were consistent with numerous observations which suggest that a small RNA molecule(s) may be an integral component of the glucocorticoid receptor and may influence activation. A multistep process for activation was actually proposed earlier when it was demonstrated that the rate of activation of glucocorticoid receptors in mouse brain cytosol varied according to which assay for activation was used (Luttge

et al., 1984a, b & d; Luttge and Densmore, 1984). Changes in binding to both DEAE-cellulose filters (ion exchange) and glass fiber filters (hydrophobic interaction) occurred at about the same rate which was twice the rate at which DNA-cellulose binding increased. Changes in sedimentation rate (indicating subunit dissociation) occurred at yet a different rate, indicating the possibility that even more than two steps may be involved in the overall activation process. It is clearly obvious that purification of the glucocorticoid receptor has greatly enhanced the understanding of the process of steroid receptor activation, but much work remains to be done in this area before final resolution of this important process has been reached.

CHAPTER V

CHARACTERIZATION OF THE UNOCCUPIED GLUCOCORTICOID RECEPTOR

Introduction

Numerous studies have revealed that glucocorticoid (as well as most other steroid) receptors are very unstable molecules in vitro, particularly when they are not bound to a steroid ligand (Kirkpatrick et al.,1972; Koblinsky et al., 1972; Bell and Munck, 1973). These receptors are stabilized considerably when they are bound by glucocorticoids (Kirkpatrick et al., 1972; Pratt et al., 1975; Rafestin-Oblin et al., 1977), and the degree of stabilization appears to be roughly proportional to the binding affinity of the steroid (Nielsen et al., 1977a,b). In light of recent reports that unbound glucocorticoid (Raaka and Samuels, 1983) and estrogen (Eckert et al., 1984) receptors appear to be relatively stable in situ (with half-lives of up to 9.5 Hr at 37 C), an understanding of those stabilizing factors that are effectively removed or diluted out during cytosol preparation would not only allow for a more accurate quantification of receptor binding data, but is virtually essential before in vitro studies of receptor up- and down-regulation can be interpreted with any degree of confidence. A number of substances added exogenously have been shown to influence the stability of unbound steroid receptors. Varying degrees of stabiliza- tion have been achieved with glycerol (Schaumburg, 1972; Korge and Timpmann,1983; Ogle, 1983), molybdate (Leach et al., 1979), sulfhydryl protecting agents (Rees and Bell, 1975; Granberg and Ballard,

1977; Sando et al., 1979; McBlain and Shyamala, 1980; Densmore et al., 1984), nucleotides (Sando et al., 1979; Barnett et al., 1983; Densmore and Luttge, 1985), and protease inhibitors (Sherman et al., 1978; Ratajczak et al., 1981; Kalimi et al., 1983). Conflicting results have been obtained with the addition of alkali salts (Schaumburg, 1972; Schmid et al., 1976; Krieger et al., 1976; Young et al., 1977; Densmore et al, 1984c), divalent cations (Schmid et al., 1976; Nakai et al., 1978; Aranyi and Naray, 1980; Ratajczak et al., 1981; Rousseau et al., 1982; Hubbard and Kalimi, 1983a,b; Kalimi et al., 1983; Densmore et al., 1984a) and various chelating agents (Bell and Munck, 1973; Rees and Bell, 1975; Schmid et al.,1976; Rafestin-Oblin et al., 1977; Ratajczak et al., 1981; Hubbard and Kalimi, 1983 a,b; Densmore et al., 1984a).

Glycerol, other polyhydric alcohols and sugars are known to increase the thermal stability of many proteins (Gerlsma and Stuur, 1972; Donovan, 1977; Back et al., 1979), including steroid receptors (Sherman, 1975). Early studies of glucocorticoid receptors reported a requirement for high concentrations (40%) of glycerol for maximum stabilization of the receptor in rat thymocyte cytosol (Schaumburg, 1972) and a similar requirement was reported for progesterone receptors from rat and mouse uteri (Feil et al., 1972), although the mechanism of glycerol stabilization was unknown. More recently, Korge and Timpmann (1983) reported that not only did glycerol stabilize dexamethasone binding in rat heart cytosol, but it also reduced markedly both the association and dissociation rate constants. It is likely that glycerol's ability to enter the layer formed in aqueous solution at the receptor surface accounts for its stabilizing effect (Ogle, 1983). It has been suggested that glycerol molecules distribute themselves

throughout the solvation sheath of proteins in accordance with the balance of forces between repulsion from nonpolar regions and attraction to the polar regions of the protein surface, as well as attraction between water and glycerol molecules (Gekko and Timasheff, 1981a). These influences tend to stabilize the more folded, or native, state of the protein and increase its chemical potential due to the preferential exclusion of glycerol from the domain of the protein. It follows then that the more hydrophobic a protein's surface is, the greater the concentration of glycerol required to enter the hydration layer and, hence, stabilize the native conformation, will be. Denaturation, or unfolding, involves an increase in the surface of contact between receptor protein and solvent, and in particular exposes additional hydrophobic residues to contact with solvent. The presence of glycerol would lead to a thermodynamically less favorable situation and require the use of more free energy for unfolding than in water. As a result, the presence of glycerol should tend to favor the folded native state of the receptor (Gekko and Timasheff, 1981a,b), possibly accounting for its increased stability at elevated temperatures as well as the additional effect of holding the ligand-binding site more rigidly in its native conformation, thus protecting high affinity binding (Ogle, 1983). In addition, the presence of high concentrations of glycerol has been shown to reduce the loss of proteins by adsorption to glass or plastic surfaces (Suelter and DeLuca, 1983) and acts as a cryoprotective agent to reduce receptor losses encountered during freezing and thawing (Densmore, see Chapter IV). One must also consider the possibility that some of glycerol's effects on receptor stability, association rates, etc., may be related to concentration-dependent changes in viscosity.

Recent work with the glucocorticoid type I receptor has indicated that glycerol has little or no effect on the stability of this receptor (Emadian et al., unpublished).

Molybdate, vanadate and tungstate are group 6A transition metal oxyanions that inhibit both alkaline (Lopez et al., 1976) and acid (VanEtten et al., 1974) phosphatases as well as some phosphohydrolases like (Na+ and K+)ATPases (Cantley et al., 1978; Karlish et al.,1979; Simons, 1979) in micromolar and submicromolar concentrations. It was for this reason that Nielsen et al. (1977a,b) added molybdate to cytosols prepared from several tissues and cultured cells where it was found to produce a profound inhibition of temperature-mediated inactivation of glucocorticoid receptors. Although molybdate's stabilizing effects were thought originally to be via a direct effect on a phosphatase enzyme (Nielsen et al., 1977a,b), several later observations provided evidence that the transition metal's inhibition of phosphatase activity and receptor activation may not be related. To begin with, the concentrations of molybdate, vanadate and tungstate required to stabilize unoccupied glucocorticoid receptors and to prevent the activation of the glucocorticoid receptor complex are generally greater (millimolar rather than micromolar) than those required for effective phosphatase inhibition (Leach et al., 1979; Wheeler et al., 1981; Gray, 1982; Densmore et al., unpublished). In addition, 10 mM molybdate reportedly did not inhibit calf intestine alkaline-phosphatase activity assayed with para-nitrophenyl phosphate as substrate, nor did it prevent inactivation of glucocorticoid-binding capacity when this enzyme was added to rat liver cytosol, although lost binding could be reactivated upon addition of a sulfhydryl protecting (reducing) reagent

such as dithiothreitol (Dahmer, 1983; Dahmer et al., 1984). In contrast, receptor inactivation occurring in the presence of reducing agents, but in the absence of transition metal oxyanions, is irreversible. These and many other observations strongly suggest that molybdate interacts directly with the receptor. Such a direct interaction has also recently been proposed to account for molybdate's similar actions on estrogen receptors (Braunsberg, 1984). This interaction is apparently a weak one because of the relatively high (millimolar) concentrations required for receptor stabilization. The interaction between the transition metals and the receptor is not likely to be the same as the interaction between vanadate and Na+, K+ ATPases since these enzymes are inhibited by several orders of magnitude lower vanadate concentration (Cantley et al., 1978). In the case of acid phosphatases, there is evidence that molybdate complexes with a histidyl residue in the enzyme active site, functioning as an analog of the transition state (VanEtten et al., 1974). Again, the concentration of the oxyanion required for inhibition of these enzymes is very low (10-1000 mM) and unlikely to represent the type of interaction occurring between molybdate and the receptor. Although the type of functional groups required for this interaction are not known for sure, it has been reported that molybdate, vanadate and tungstate can form chelates with a variety of functional groups, including oxygen, nitrogen and sulfur groups (VanEtten et al., 1974). Kay and Mitchell (1968) reported the formation of molybdenum-cysteine complexes and suggested that such interactions may be involved in molybdenum-containing enzymes. Weathers et al. (1979) have also reported that molybdenum ions have a high affinity for sulfur groups and, in fact, appear to bind proteins via

thiol group interactions, which in itself may be sufficient to stabilize proteins (Brandt and Andersson, 1976). It has been suggested that binding of the molybdate ion to thiol groups may form a molybdenum bridge between receptor subunits (Ogle, 1983; Renoir and Mester, 1984; Wilson et al., 1986). A more detailed understanding of this interaction would likely provide valuable clues as to the actual molecular mechanisms involved in receptor activation, in vivo stabilization and, possibly, mechanisms of receptor up and down regulation.

Although molybdate has clearly been shown to inhibit activation of bound receptors and irreversible loss of binding capacity in unoccupied receptors, Mendel et al. (1985), by analyzing parallel cytosol samples under denaturing conditions, have been able to demonstrate that the stability of the nonactivated glucocorticoid receptor complex in the presence of molybdate is only apparent. These workers reported that while gel filtration chromatography showed that molybdate does "stabilize" the nonactivated complex, so that after 2 hr at 3 C it remained as a multimer with Stokes radius of about 8 nm; SDS-PAGE, by contrast, indicated that this complex is made up primarily of 50,000 dalton fragments instead of intact 90,000 dalton monomers. Degradation of the receptor to this size did not, according to these workers, affect the ability of the complex to undergo cell-free activation. To determine if the effects of molybdate on steroid receptor stability and activation in vitro reflect the nature of molybdate-receptor interactions in vivo, Raaka et al. (1985) examined the effects of treating intact GH1 cells with molybdate and reported an inhibition of the subsequent rate of nuclear accumulation of hormone-occupied glucocorticoid and estrogen receptors. These workers also reported that

although molybdate did not affect the rate of receptor occupancy with either steroid, cells treated with molybdate had more occupied cytosolic and fewer occupied nuclear receptors than control cells. Svec (1985b), however, reported that neither activation nor nuclear translocation of glucocorticoid receptors was affected in vivo when intact AtT-20 mouse pituitary tumor cells were incubated with molybdate.

Okret et al. (1985) reported evidence that the molybdate-stabilized glucocorticoid receptor is actually a heteromeric complex of approximately 302,000 daltons consisting of a single 94,000 dalton steroid-binding subunit and other non-steroid-binding subunit(s) which apparently dissociate during activation. More recently, Mendel et al. (1986) reported that a 90,000 dalton non-steroid-binding phosphoprotein, which is lost upon activation, is associated with the molybdate-stabilized glucocorticoid receptor from WEHI-7 mouse thyoma cells. Since the activation-associated dissociation of these heterologous subunits is greatly facilitated by, and probably dependent upon, ligand binding, a more appropriate way of studying the native form of the receptor in cytosol would be to use the non-liganded form, both in the presence and in the absence of molybdate.

Materials and Methods

Chemicals, Steroids and Isotopes

[6,7-3H]Triamcinolone acetonide, or 9a-fluoro-11b,16a,17a,21-tetra-hydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide, ([3H]TA, specific activity = 37 Ci/mmol) and [6,7-3H]dexamethasone, or 9a-fluoro-16a-methylprednisolone ([3H]DEX, specific activity = 44.1-48.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Sephadex G-25 (fine) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ).

Dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) was courtesy of Research Organics (Cleveland, OH). Sodium molybdate (Na2MoO4), calf thymus DNA-cellulose, glycerol, sucrose, PPO (2,5-diphenyloxazole) and dimethyl POPOP (1,4-bis[2(4-methyl-5-phenyl-oxazoyl)]benzene) and pentyl agarose were all purchased from Sigma Chemical Co. (St. Louis, MO). Scinti Verse II was purchased from Fisher, Inc. (Fair Lawn, NJ). All other chemicals and solvents were reagent grade.

Animals

All studies used female CD-1 mice (Charles River Laboratories, Wilmington, MA) that were subjected to combined ovariectomy and adrenalectomy approximately 1 week prior to each experiment in order to remove known sources of endogenous steroids. Both operations were performed bilaterally via a lateral, subcostal approach under barbiturate anesthesia, and mice were given 0.9% NaCl (w:v) in place of drinking water. On the day of the experiment mice were anesthetized with ether and perfused slowly through the heart with ice-cold HEPES-buffered saline (20-30 ml, isotonic, pH 7.6).

Cytosol Preparation and Steroid Binding

Brains and other tissues were removed from the perfused animals and homogenized (2x10 strokes at 1000 rpm) in 4 volumes of ice cold buffer containing 20 mM HEPES and 2 mM DTT, pH 7.6 at 0 C in a glass homogenizer with a Teflon pestle milled to a clearance between the pestle and homogenization tube of 0.125 mm on the radius (to minimize rupture of the brain cell nuclei (McEwen and Zigmond, 1972). The crude homogenate was centrifuged at 100,000 g for 20 min and the supernatant recentrifuged at 100,000 g for an additional 60 min to yield cytosol.

During these centrifuge runs, and during all other procedures, unless otherwise indicated, careful attention was paid to maintaining the cytosol at 0-2 C. Final protein concentrations were typically in the 6-8 mg/ml range. For prelabeled receptor preparations, cytosol was incubated in the presence of 20 mM molybdate with 20 nM [3H]TA or [3H]DEX for 24 to 40 hours at 0 C with or without a 200-fold excess of unlabeled steroid.

Preparation of Heat-stable Cytosolic Factors

Brain or liver cytosol (prepared as above) was incubated in a boiling water bath for 10 min and precipitated proteins were removed by centrifugation at 100,000 g for 20 min.

Sucrose Density Gradient Sedimentation

Aliquots (400 ul) of either unlabeled or prelabeled cytosol were layered onto linear 5-20% sucrose density gradients (4.6 ml; prepared with HEPES buffer containing 2 mM DTT and either with (for molybdate-stabilized receptors) or without (for nonmolybdate-stabilized receptors) 20 mM molybdate and centrifuged at 0 C for 1.5-2 hr at 370,000 g (average) in a Sorvall TV-865 vertical tube rotor. The cellulose nitrate tubes were punctured and 26-28 fractions (180 ul) collected and incubated with 20 nM [3H]DEX +/- 4 uM [1H]DEX in the presence of 20 mM molybdate at 0 C for 24 hr. The individual fractions were then assayed for specific binding. Sedimentation coefficients (S20,w) were calculated from the linear regression of S20,w vs sedimentation distance (Martin and Ames, 1961) for the following standard proteins run in parallel tubes: chicken ovalbumin (OVALB, 3.6 S), bovine serum albumin (BSA, 4.3 S), bovine gamma globulin (IgG, 7.4 S) and catalase (CAT, 11.3 S). The standard proteins were

[14C]methylated (for detection) to low specific activity with [14C]formaldehyde by the method of Rice and Means (1971).

Steroid Binding Determination

In all experiments, bound 3H-steroid was separated from free on Sephadex G-25 columns (0.6 x 14 cm) pre-equilibrated in buffer identical to that in which the cytosol was prepared. Duplicate aliquots from each assay tube (BT and BNS) were layered onto separate columns, allowed to penetrate the gel and the macromolecular (bound) fraction eluted (with homologous buffer) directly into scintillation vials for liquid scintillation spectrometry.

Results

A major problem in the early studies of glucocorticoid, as well as most other steroid, receptors before the discovery of molybdate's effects on receptor complex activation was that activation was often occurring throughout many, if not all, of the experimental steps, frequently rendering the results uninterpretable. Never-the-less, attempts were often made to interpret these results with little or no concern for the impact of uncontrolled receptor activation. The principle effect of molybdate on unoccupied glucocorticoid receptors, perhaps via a mechanism similar to its inhibition of steroid-receptor complex activation, is to inhibit the irreversible inactivation of glucocorticoid binding capacity. This receptor lability is greatly enhanced when the receptor is unoccupied by steroid ligand and even more so when cytosolic stabilizing factors are separated from the receptor (either intentionally or not) by any number of analytical procedures. As a consequence, uncontrolled instability generally results in no data at all, instead of confounded data that is likely to be misinterpreted.

For this reason and because of the fact that many physicochemical studies of unoccupied receptors require a complicated postlabeling procedure that would not be required for studies of prelabeled receptors, few studies have been conducted on the physicochemical properties of glucocorticoid receptors and virtually none of these have been carried out in the absence of molybdate. The initial experiments in this series sought to determine if the overall size and shape of the unoccupied receptor, as determined by sedimentation analysis, was the same or different from that of the occupied unactivated receptor. The first experiment used the traditional swinging bucket rotor method which typically requires a 16 to 24 hr ultracentrifugation. Because of the long centrifuge times, this experiment could only be carried out using sucrose gradients containing 20 mM molybdate. The results indicated that the molybdate-stabilized unoccupied receptor was indeed of the same sedimentation coefficient as the molybdate-stabilized unactivated glucocorticoid receptor (see Chapter III, Figure 3-4). These same results also revealed that sulfhydryl modification of the molybdate-stabilized unoccupied receptor in or around the steroid-binding site, an action of potential physiological relevance, had no effect on sedimentation characteristics. Hydrophobic interaction chromatography was also used to compare the properties of the occupied and unoccupied glucocorticoid receptors (see Chapter II, Figure 2-6), but again the procedure (because of high ionic strength requirements) required the use of molybdate-stabilized forms and again the binding of steroid ligand was found to be ineffectual in altering surface hydrophobicity properties.

A relatively recent modification of sedimentation analysis that has markedly reduced the centrifugation time required involves the use of a vertical tube rotor (for a discussion of vertical tube sedimentation analysis of steroid receptors, see Chapter IV). Since the use of a vertical tube rotor can decrease the centrifugation time by an order of magnitude or better, the sedimentation properties of unoccupied receptors in both molybdate- and non-molybdate-containing sucrose gradients (5-20%) was investigated using such a rotor. In the same run were included unactivated [3H]TA-labeled glucocorticoid receptor complexes also run on molybdate-containing gradients for direct comparative purposes. The 8 position rotor allowed each of these groups to be run in duplicate along with protein standards run on both molybdate-containing and non-molybdate-containing gradients. After a two hour centrifuge run at 0 C, the non-molybdate-containing gradients, because of their high lability, were removed first and rapidly fractionated into test tubes containing 20 nM [3H]TA and then incubated at 0 C for 24 hr. The unoccupied receptors sedimented on molybdate-containing gradients were next fractionated in an identical fashion and incubated with [3H]TA as well. The gradients containing prelabeled receptors and [14C]-labeled protein standards were fractionated directly into scintillation vials for counting.

As expected, 20 mM molybdate had no effect whatsoever on the elution profiles of the standard proteins. The prelabeled unactivated receptor sedimented at the rate predicted (9 S) based on previous findings by this lab using the more traditional swinging bucket rotors (Luttge et al., 1984a-d; Luttge and Densmore, 1984). After bound/free steroid separations had been performed using Sephadex G-25

chromatography on the individual postlabeled fractions from the unoccupied receptor gradients, it was evident that the molybdate-stabilized form of the unoccupied glucocorticoid receptor sedimented at precisely the same rate that the molybdate-stabilized prelabeled unactivated glucocorticoid receptor complex did. Unfortunately, the sucrose gradients containing the non-molybdate-stabilized unoccupied receptors exhibited no binding activity whatsoever. This finding was unexpected since although unoccupied glucocorticoid receptors are much less stable than their steroid-bound counterparts, they are generally not this labile, even in the absence of molybdate, when low temperature is maintained. Obviously the process of centrifugation must have been removing some cytosolic factor(s) responsible for stabilizing the unoccupied receptor from irreversible degradation that is prevented by the presence of molybdate.

The next attempt at characterizing the unoccupied glucocorticoid receptor in the absence of molybdate required a number of modifications of the vertical rotor sedimentation procedure and the subsequent steroid incubation step. One change involved the precooling of the vertical rotor to approximately -4 C prior to the centrifuge run. Previous runs had been made with a rotor prechilled to approximately 4 C and although the centrifuge run was made at 0 C, the temperature of the rather massive vertical rotor was likely to have remained well above 0 C for the 2 hr under the high vacume required for a 65,000 rpm spin. The low temperature of -4 C was considered warm enough to prevent the possibility of freezing of the sucrose gradients during the centrifuge run which would seriously disrupt, if not destroy, the resolution of the gradient. Another corrective measure taken was the inclusion of 20 mM molybdate in

each of the postlabeling incubation tubes. This addition of molybdate after the fractionation of the non-molybdate-containing gradients would not alter the sedimentation profile of the non-molybdate-stabilized form, but would act to prevent much of the receptor degradation occurring after gradient fractionation and during the steroid incubation period. A final modification to the procedure involved the inclusion of KCl in the sucrose gradients. It had been shown previously that the addition of a number of monovalent cations (i.e. Na, K, Li, Cs and Rb) to cytosol dramatically increased the thermal stability of non-molybdate-stabilized unoccupied receptors (Densmore et al., 1984a). Since small molecules including these cations are quickly separated from the relatively fast-sedimenting receptor early in the centrifugation run, the receptor is suddenly forced into an environment of lower ionic strength than exists in cytosol. To counteract the possibility of low ionic strength-induced destabilization of non-molybdate-stabilized receptors, 2 different concentrations of KCl were introduced into the gradients including 50 and 150 mM. Protein standards were run on gradients containing each of these concentrations of KCl and unoccupied receptors were also run on gradients containing 20 mM molybdate and 150 mM KCl. The postlabeling of unoccupied receptors was carried out as previously described except for the addition of molybdate to the incubation tubes of the non-molybdate-stabilized form.

As was the case with the presence or absence of molybdate, the sedimentation profiles of the protein standards were not affected by the addition of either 50 or 150 mM KCl. Likewise, the unoccupied receptors run on molybdate-containing gradients displayed the same sedimentation properties when run in the presence or absence of 150 mM KCl. Finally,

the precautionary measures taken to reduce the degree of degradation of unoccupied receptors when run on gradients lacking molybdate appeared to have been successful. A major peak was evident at 9 S, or precisely the same position as the single peak exhibited by the molybdate-stabilized unoccupied and steroid-bound forms (Fig. 5-1). A minor peak sedimenting at 4-5 S was also apparent in all of the profiles (50 mM and 150 mM KCl, both run in duplicate). Although the positions of both peaks were highly replicable across all 4 gradients, the total quantity of viable receptors measured from these gradients was only a small fraction of what was measured from molybdate-containing gradients on which equal aliquots from the same pool of cytosol were run.

In order to further stabilize both peaks sedimenting on the non-molybdate gradients, an effort was made to isolate whatever factor(s) present in cytosol were responsible for stabilizing the unoccupied receptor. The first experiment sought to determine whether or not these factors were macromolecular or micromolecular. Cytosol prepared in the absence of molybdate was simply run on a small Sephadex G-25 column equilibrated and eluted with non-molybdate containing buffer. The macromolecular fraction was then incubated with 20 nM [3H]DEX +/- 4 uM [1H]DEX for 24 hr at 0 C. A similar aliquot of non-gel-filtered cytosol from the original pool was incubated likewise. After bound/free separations were performed on Sephadex G-25 columns and dilutions occurring as the result of the initial gel-filtering of unlabeled cytosol were taken into account, it was determined that removal of small molecules led to a 90% reduction in binding capacity (data not shown).

Figure 5-1. Vertical tube rotor sucrose density gradient sedimentation analysis of nonmolybdate-stabilized unoccupied Type II glucocorticoid receptors on nonmolybdate-containing sucrose gradients. Brain cytosol was prepared in HEPES buffer containing only 2 mM DTT. Aliquots (400 ul) were applied to 4.6 ml, prechilled, 5-20% linear sucrose gradients (made in the absence of molybdate) which were then placed in a TV-865 vertical tube rotor prechilled to approximately -2 C and run at 65,000 rpm (370,000 x g (average)) for 2 hr. Tubes were then quickly fractionated into steroid incubation tubes containing HEPES buffer plus molybdate (20 mM final concentration) and DTT (2 mM final concentration) and 20 nM [3H]TA. After a 40 hr incubation, bound-free steroid separations were performed on Sephadex G-25 columns. Binding is expressed as percent of the total counts (mean > 1600 cpm) collected from each gradient and represents the mean value of the same fraction from three gradients. The profile shown here is representative of 4 independent replications.

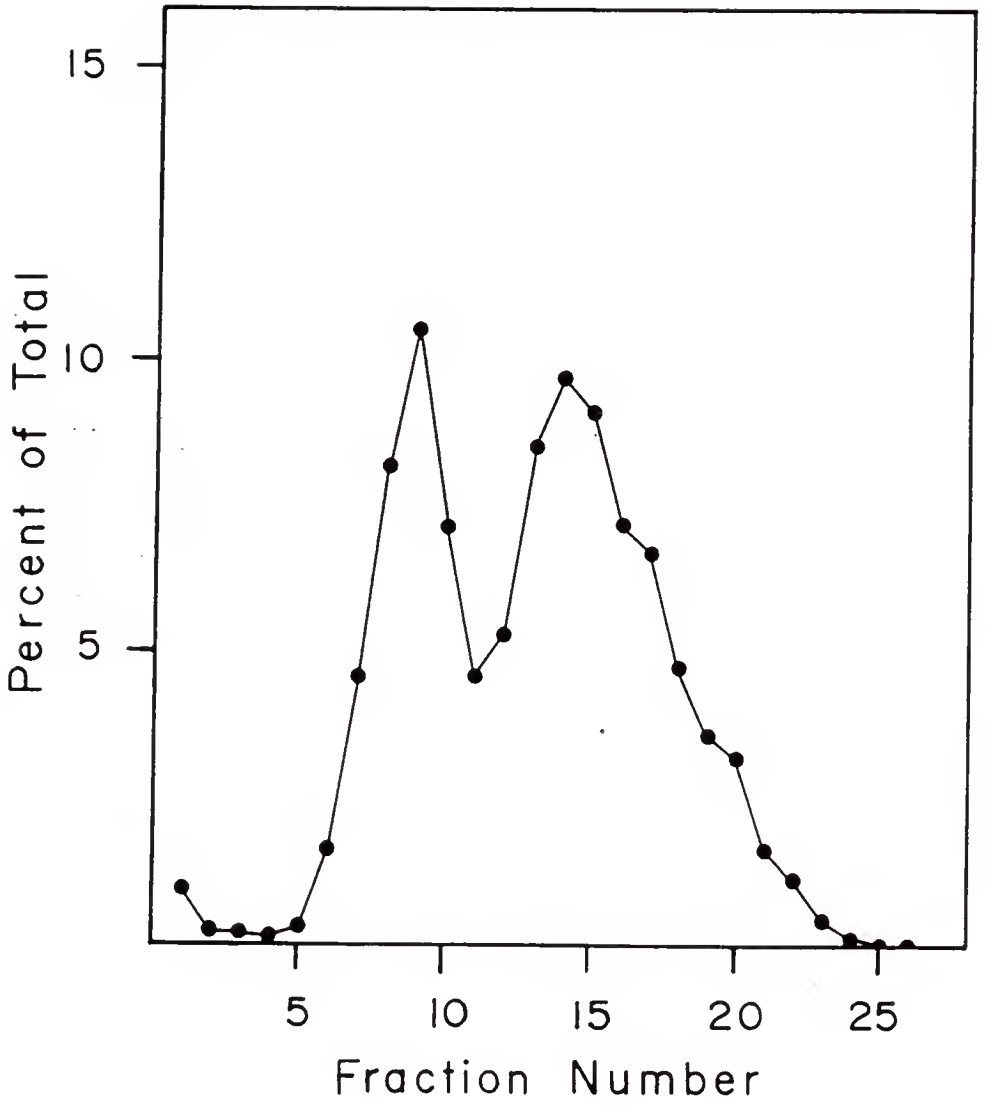
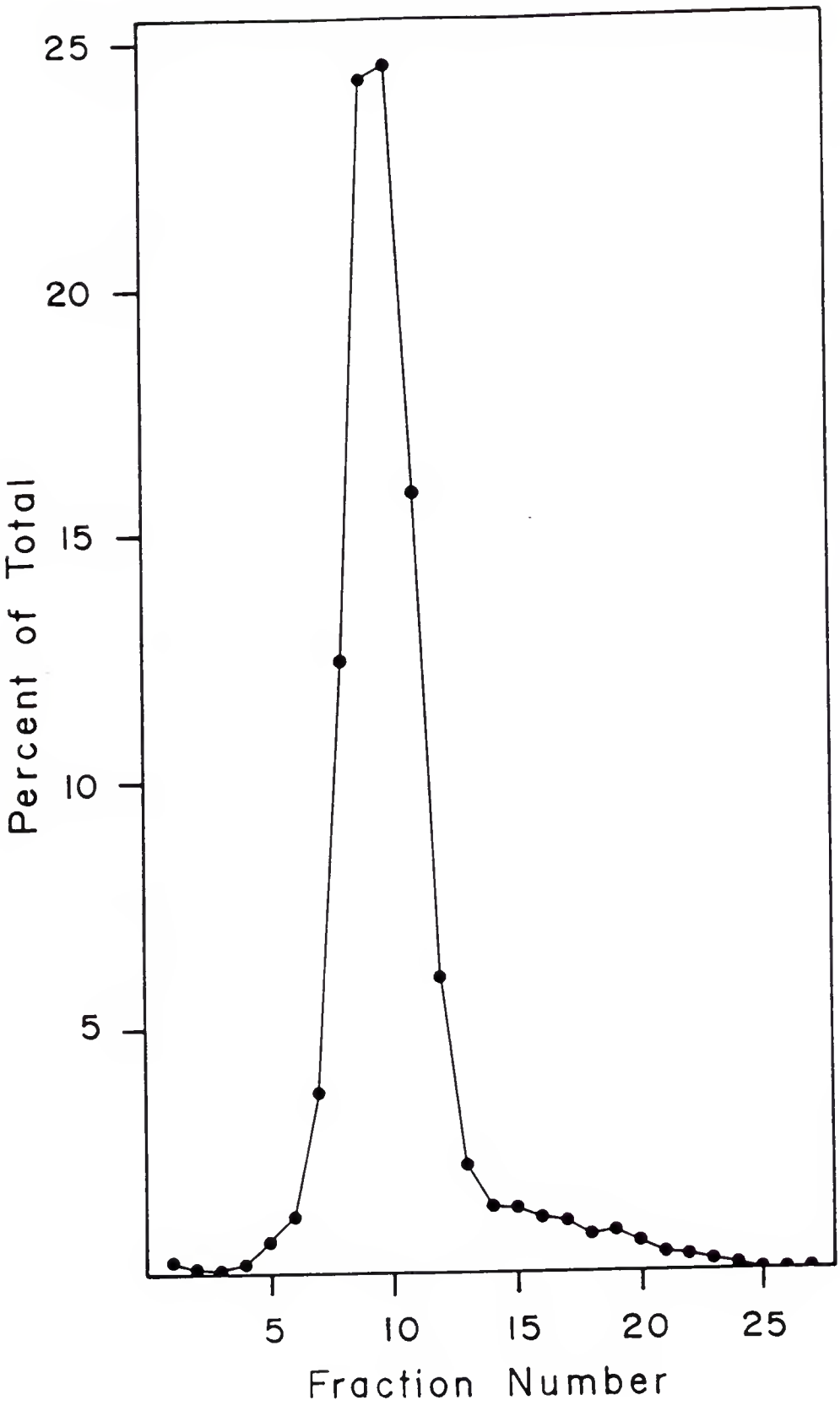

15
10
5
Percent of Total
5
10
15
20
25
Fraction Number

Figure 5-2. Vertical tube rotor density gradient sedimentation analysis of the molybdate-stabilized unoccupied Type II glucocorticoid receptor. Brain cytosol was prepared in HEPES buffer containing 20 mM molybdate and 2 mM DTT. Aliquots of cytosol (400 ul) were applied to 5 ml, prechilled, 5-20% linear sucrose density gradients (made in the presence of molybdate) and then run in a TV-865 vertical tube rotor at 65,000 rpm (370,000 x g (average)) for 2 hr. The tubes were then quickly fractionated into steroid incubation tubes containing 20 nM [3H]TA. After a 40 hr incubation, bound-free steroid separations were performed on Sephadex G-25 columns. Fractions are represented as percent of total counts (12,000 cpm) collected from the gradient. The profile shown here is representative of 4 independent replications.


25
20
15
10
5
Percent of Total
5
10
15
20
25
Fraction Number

The next step was to determine if the factor(s) were heat stable or heat labile. For this experiment, brain cytosol was prepared as previously described in the absence of molybdate. A portion of the unlabeled cytosol was retained on ice while the remainder was incubated in a boiling water bath for 10 min. The heat-treated cytosol was then subjected to centrifugation at 100,000 g for 15 min to remove any precipitated proteins, etc.. The resulting supernatant was then used to equilibrate small Sephadex G-25 columns of the same dimensions as those used in the previous experiment for macromolecular/micromolecular separation. Other identical columns were equilibrated in either non-molybdate-containing buffer or molybdate-containing buffer. Aliquots of the non-heat-treated cytosol were applied to each of these columns and eluted with the corresponding buffer, heat-treated cytosol preparation, etc.. The macromolecular fractions were collected from these columns and either incubated directly with 20 nM [3H]DEX +/- 4 uM [1H]DEX for 24 hr at 0 C, or first incubated for 30 min at 20 C prior to the steroid incubation.

The binding of the preparations run through the non-molybdate columns were, on average, only about 10% of those run through molybdate-equilibrated columns, whereas those preparations run through columns equilibrated with heat-treated cytosol retained nearly 70% of the maximal binding. Of those preparations that were incubated at 20 C prior to steroid incubation, the molybdate-treated group displayed no loss in binding, whereas the binding of the non-molybdate treated group was reduced by more than 80%. Preparations run through columns equilibrated with heat-treated cytosol were, by comparison,

reduced by only 50%, indicating an obvious protective effect of the heat-treated cytosol fraction.

A more extensive experiment was conducted to further investigate the ability of these heat-stable factors to stabilize the unoccupied receptor in vitro. In this experiment, cytosol from both brain and liver (equal wet weights of each tissue were used) was prepared in the previously described manner using non-molybdate containing buffer. A portion of each of these cytosol preparations was incubated in a boiling water bath for 10 min followed by centrifugation at 100,000 g for 10 min. Non-heat-treated brain cytosol was run on a series of columns equilibrated and eluted with either molybdate-containing or non-molybdate-containing buffer. The macromolecular fraction (800 ul) was collected into tubes containing 800 ul of one of the following: molybdate-free buffer, molybdate-containing buffer, 100% heat-treated liver cytosol, 50% heat-treated liver cytosol and 50% molybdate-free buffer, 25% heat-treated liver cytosol and 75% molybdate-free buffer, 100% heat-treated brain cytosol, 50% heat-treated brain cytosol and 50% molybdate-free buffer, 25% heat-treated brain cytosol and 75% molybdate-free buffer, the macromolecular fraction of heat-treated liver cytosol and the macromolecular fraction of the heat-treated brain cytosol. The collection tubes were maintained on ice during the filtration process. An aliquot of the contents of each group was incubated directly with 20 nM [3H]DEX +/- 4 uM [1H]DEX while a second aliquot was first incubated for 30 min at 20 C prior to steroid incubation.

The results from this experiment again indicated a protective effect of heat-treated cytosol, but not the macromolecular fraction of

heat-treated cytosol. Heat-treated liver cytosol was more effective than heat-treated brain cytosol and the effect appeared to be concentration dependent. The degree of binding loss was greater in this experiment when heat-treated cytosol was added to the gel-filtered macromolecular fraction of non-heat-treated cytosol than it was in the previous experiment where the Sephadex G-25 columns were actually pre-equilibrated with the heat-treated cytosol. This difference could either be attributed to the increased dilution of the heat-stable factor when added after gel filtration of non-heat-treated cytosol or degradation occurring in the absence of the heat-stable factor during the column run itself.

The findings of the previous experiment led to the modification of the procedure described previously for vertical tube rotor sedimentation analysis of non-molybdate-stabilized unoccupied glucocorticoid receptors. Most of the precautionary steps that had been added to increase receptor stability such as pre-chilling of the rotor and post-fractionation addition of molybdate were retained. The sucrose gradients, however, were made up with 50% heat-treated liver cytosol prepared as previously described.

The results of this experiment indicated that while the major peak (the peak previously sedimenting at 9 S) was still apparent in these gradients, the smaller peak was not, indicating that the presence of the heat-stable factor(s) may have been having an effect on the presence of the different sedimenting forms of the unoccupied receptor similar to the effect of molybdate. The total bound radioactivity resulting from these gradients was still significantly lower than that obtained with gradients containing molybdate. Not surprizingly, aggregation appeared

to be a little more of a problem than had previously been encountered with gradients lacking the heat-treated cytosol.

Discussion

Before the discovery that molybdate was an effective inhibitor of inactivation of unoccupied glucocorticoid receptor binding capacity and activation of the receptor to a nuclear-binding form (Leach et al., 1979), characterization of the unoccupied receptor was virtually impossible because of the greater lability of this form of the receptor. This was particularly evident after gel filtration or other analytical procedures that tended to separate the unoccupied receptor from the cytosolic factor(s) that appear to provide some degree of stabilization. As a consequence, virtually all reports published on the physicochemical properties of glucocorticoid receptors employed the use of prelabeled receptors, which reduced the problem of lability while introducing the problem of uncontrolled activation of receptors during the often lengthy analytical procedures. Unoccupied receptors, in the absence or presence of molybdate, appear incapable of undergoing activation prior to the binding of a steroid ligand (Atger and Milgrom, 1976; Bailly et al., 1978; Densmore, unpublished). This implies either that binding of the steroid illicits conformational changes in the receptor molecule prerequisite for the subsequent steps of activation or that if the unoccupied receptor does undergo an activation of sorts, it apparently renders the receptor inactive and incapable of binding steroid. Strong evidence for the latter of these possibilities has most recently been provided with the finding that activated glucocorticoid receptors appear completely incapable of rebinding steroid after steroid dissociation, even in the presence of molybdate (Chou and Luttge, unpublished).

Although the use of molybdate for receptor stabilization has become an almost routine part of most steroid receptor studies, the vast majority of reports still focus on the characteristics of prelabeled receptors. Only one report has examined the effects of molybdate on the sedimentation properties of non-liganded steroid receptors. El Dieb et al. (1983) described the effect of ligand binding on the sedimentation behavior of cytosolic progestin receptors. They found that when cytosol was prelabeled, receptors sedimented at 4.4 S and 7.8 S, whereas when the fractionated gradients were postlabeled, the unoccupied receptors sedimented at 4.4 S and 9-10 S. They also noted that 20 mM molybdate blocked the conversion of 9-10 S to 7-8 S receptors. In contrast with the results for unoccupied glucocorticoid receptors in the present study wherein the 4-5 S peak for the unoccupied glucocorticoid receptor was only apparent on molybdate-free gradients, the 4.4 S peak for progestin receptors was reportedly not eliminated by the presence of molybdate in the gradient. Despite the similarities in amino-acid structure between progestin and glucocorticoid receptors (Conneely et al., 1986), other tissue, species, buffer and methodology differences between the two studies make a direct comparison of the results difficult.

A more recent study has examined the chromatographic behavior of non-liganded glucocorticoid receptors in the presence and absence of 20 mM molybdate on Agarose A-1.5 m columns (Radojcic et al., 1986). In the absence of molybdate, a single peak corresponding to a Stokes radius of 5.7 nM was detected, while addition of molybdate throughout the course of the chromatographic analysis of non-liganded receptor resulted in the appearance of a single peak with a Stokes radius of 8.0 nM. While these authors' results are similar to those obtained in the present study

using sedimentation analysis, Radojcic et al. (1986) detected non-liganded receptors post-procedure with an enzyme-linked immunosorbent assay (ELISA) based on antibodies raised in rabbits against the purified activated glucocorticoid receptor. Unfortunately, these workers did not show whether or not the peaks they detected were actually still capable of binding steriod, thereby eliminating the possibility that the antibody was merely recognizing an inactive degradation product of the receptor.

The ability of heat-stable factors to eliminate the appearance of the 4.5 S-sedimenting form of the unoccupied glucocorticoid receptor in the present study is indicative of several possibilities. One or more heat-stable cytosolic factors may be acting much like molybdate, but in a more limited fashion, to hold receptor subunits together. If this is the case, the slow-sedimenting form appearing on gradients not containing either molybdate or heat-treated cytosol may represent a nonphysiological artefact of the procedure. Another possibility is that the slower sedimenting form exists in vivo, but is rapidly inactivated in vitro by heat-stable factors. An understanding of this phenomenon may eventually enhance our knowledge of the apparent heterogeneous subunit makeup of the glucocorticoid receptor in vivo and what role the individual subunits play in the overall mechanism of glucocorticoid receptor-mediated action. The recent suggestion that one or more of these subunits, in particular a 59,000 dalton non-steroid-binding subunit, is similar or identical to subunits found in unactivated progestin, estrogen and androgen receptors (Tai et al., 1986), indicates the significance of the findings of the present study to an understanding of steroid receptor mechanisms in general.

CHAPTER VI

CONCLUDING REMARKS

The work of this dissertation focussed on a number of different questions regarding the structure and function of the glucocorticoid type II receptor in mammalian brain. While some of these questions may at first seem to be disconnected, it is important to realize the relation that they have to one another and to an overall understanding of the genomic mechanisms of glucocorticoid action. A complete understanding of these genomic mechanisms requires a thorough understanding of the glucocorticoid receptor, a necessary intermediate in the overall scheme. In turn, anything that affects the receptor's essential role in the process must likewise be understood. Clearly, anything that affects the ability of a receptor to bind steroid, the rate of steroid association, the rate of receptor activation to its nuclear binding form, the ability of the activated form to bind to its nuclear acceptor site(s) or the stability of any or all of these forms could potentially have an impact on the cell's ability to respond to a glucocorticoid hormone signal. It may therefore not be completely sufficient to know only the concentration of glucocorticoid receptors in a given tissue, in order to predict precisely how sensitive that tissue will be to glucocorticoids under particular physiological conditions. Obviously it is impossible in one study to consider every factor that could impact on receptor-mediated glucocorticoid actions and it is certainly a difficult task to apply, with a high degree of confidence,

the knowledge gained from in vitro studies using crude, incomplete and decompartmentalized preparations to a complex, highly ordered living system, but such studies are nevertheless essential in order to complete the framework for such a global understanding of glucocorticoid action. In addition, because of very recent findings that other steroid receptors, particularly those binding progesterone (Conneely et al., 1986), have amino acid sequences that are remarkably similar to that of the glucocorticoid receptor, it is not difficult to imagine that some, if not much, of what is learned regarding glucocorticoid receptor function could be applied to the understanding of other steroid receptor systems in other tissues and other organisms spanning a vast phylogenetic realm.

One aspect of the work involved an in depth investigation of the surface hydrophobicity of the various forms of the glucocorticoid receptor. It is surprizing that although the intracellular localization of activated, unactivated and unoccupied glucocorticoid receptors in vivo is currently a controversial topic, little or no attention has been paid to possible hydrophobic interactions between various receptor forms and cellular components. While a great deal of attention has been focussed on the ionic characteristics of glucocorticoid and other steroid receptors, there is little doubt that hydrophobic properties may also play an important role in the cellular compartmentalization of these receptors. This study found that activation of the glucocorticoid receptor was associated with a profound increase in surface hydrophobicity as determined by hydrophobic interaction chromatography. This increased hydrophobicity could be indicative of changes required for the glucocorticoid receptor molecule to effectively interact with the molecular machinery that controls gene expression.

Another in vitro phenomenon which could potentially represent a mechanism by which glucocorticoid action is affected in vivo has to do with reversible changes in the glucocorticoid receptor's binding status. The reduction of glucocorticoid binding capacity associated with sulfhydryl oxidation and the restoration of the lost binding capacity associated with sulfhydryl reduction was examined in some detail. Because of the typically nonspecific nature of many sulfhydryl reactions, the incredibly complex system of intra- and extracellular thiols and disulfides and the myriad of enzymes that catalyze protein sulfhydryl oxidation and reduction reactions in a relatively nonspecific fashion, precise conclusions regarding the role of such a mechanism in the in vivo up- and down-regulation of glucocorticoid binding capacity simply cannot be made. For that matter, similar conclusions also cannot be made regarding the in vivo role of sulfhydryl oxidation and reduction in regulating the enzyme activity of any thiol-containing enzyme or the ligand binding activity of any thiol-containing receptor for basically the same reasons. Never-the-less, there exists a remarkable array of enzymes and receptors whose activities can be readily turned up or down, on or off by experimentally altering the thiol/disulfide ratio of the system, implying the possibility, at least, that many of these molecules, including glucocorticoid and other steroid receptors, may exist in vivo in a reversibly inactivated form. Even if these inactive or "down-regulated" forms exist in some sort of constant equilibrium with the active or "up-regulated" forms in the absence of an active mechanism for shifting the equilibrium in response to physiological needs, the implications for sustained glucocorticoid sensitivity of

target tissues during abnormal (or perhaps even normal) exposure to steroids are obviously worth consideration. Results from this study have revealed a number of similarities as well as differences in the biochemical and hydrodynamic properties between the sulfhydryl up- and down-regulated receptors which could have functional significance if both forms are someday unambiguously proven to exist in vivo. The sedimentation properties of the two forms are identical, indicating that down-regulation of the receptor is not likely to involve mixed disulfide formation between the receptor and another protein subunit. Hydrophobic interactions, on the other hand, are increased slightly as a result of sulfhydryl oxidation. Such differences could potentially affect subcellular compartmentalization, receptor stability, etc. in vivo. Biochemical differences include the susceptibility of up-regulated, but not down-regulated, forms to irreversible inactivation by sulfhydryl reactive reagents such as NEM, providing a potential means of measuring the relative populations of up- and down-regulated forms in homogenate or cytosol with a higher degree of certainty. This is because subsequent in vitro oxidation or reduction of up- or down-regulated receptors respectively is prevented during the long steroid incubations required to determine binding capacity.

Activation of the glucocorticoid receptor is a topic that has received a great deal of attention in the field for many years, yet the mechanisms of activation remain shrouded in controversy. This is partially the result of years of describing activation in terms of physicochemical changes in receptor properties associated with the process while not controlling for activation during the analytical procedures. In addition, activation now appears to be a multistep

process dependent, in part, on a number of cytosolic factors or conditions which typically vary from one lab's preparation to another's. Although the discovery that molybdate inhibits the process of activation has greatly aided the study of this phenomenon, there are many in the field who feel that the molybdate-stabilized receptor may not be representative of the in vivo unactivated form. Results from this dissertation have provided some information relevant to both an understanding of the process of activation as well as an understanding of the structure of the non-molybdate-stabilized unactivated receptor. The former was accomplished by perfecting a purification procedure for the glucocorticoid receptor and the latter involved perfecting analytical procedures for the highly labile unoccupied receptor not stabilized by cytosolic factors or molybdate. Whereas heat activation of the purified glucocorticoid receptor led to changes similar to those exhibited by unpurified receptors, such as increased hydrophobicity, the purified receptors showed no concomitant increase in affinity for DNA. The dependence of the receptor on cytosolic factors in order to achieve complete and effectual activation introduces yet another possible point at which the cell can influence genomic glucocorticoid actions. With regards to the structure of the unactivated receptor, there appears to exist a slower sedimenting form, when unoccupied receptors are run in the absence of molybdate. This form disappears, however, if small molecular weight cytosolic factors are present in the gradient, implying that the molybdate-stabilized unactivated receptor may be representative of the in vivo unoccupied and occupied unactivated receptor after all.

In conclusion, the findings presented in this dissertation provide new details regarding several interrelated steps in the receptor

mediated process of glucocorticoid action. Though the work is a testament to the incredibly complex task of determining potential mechanisms of steroid receptor regulation and activation, the complexity of the system makes the eventual elucidation of glucocorticoid receptor structure, function and regulation an even more important event in the overall realm of cell biology and biochemistry, not to mention its importance to understanding the more specific role of glucocorticoids in the function of the nervous system.

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BIOGRAPHICAL SKETCH

The author was born June 30, 1954 in Birmingham, Alabama, where he lived for the first five years of his life. At age five, his family moved to beautiful Huntsville, Alabama, home of the Redstone Arsenal and the Marshall Space Flight Center, at a time when the U.S. space program was just developing. The high tech, "space age" environment of Huntsville played an important role in shaping the dreams and aspirations of the author during most of the next twenty years. Upon graduating from S. R. Butler High School in 1972, the author enrolled at the University of Alabama in Huntsville where he earned his B.S. degree in biology. Because of the preponderance of aerospace industries and the author's general interest in space science, he took a position with a local aerospace firm upon leaving college. During the next two years his experience included projects related to the Space Lab/Space Shuttle program (NASA), early phases of the Strategic Defense Initiative (U. S. Army), particle accelerator electromagnet construction (Brookhaven National Laboratory) and numerous other aerospace efforts. During this time he also started graduate studies in the Department of Biological Sciences at the University of Alabama in Huntsville under the supervision of Dr. Harold J. Wilson. He eventually took a graduate teaching assistantship position with the Department and later earned a Master of Science degree. In 1979 he married Anita Anna Tiller and in 1980 they moved to Gainesville, Florida, where they both began graduate studies at the University of Florida. The author conducted his doctoral

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Neuroscience

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